TEL AVIV UNIVERSITY

DEPARTMENT OF MICROBIOLOGY

ADHERENCE OF BACTERIA TO HYDROCARBONS AND OTHER HYDROPHOBIC SURFACES

Thesis submitted for the degree "Doctor of Philosophy"

by

MEL ROSENBERG

Submitted to the Senate of Tel Aviv University

February, 1982

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This work was carried out under the supervision of
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To Shuli,

my parents and hers

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INTRODUCTION

Although the ability of microorganisms to adhere to surfaces and partition at interfaces has long been recognized and studied (73,74,123), only in the past decade has the field of bacterial adherence gained wide recognition and generated extensive scientific investigation. One sign of the increasing interest in bacterial adherence phenomena is the number of books recently published which deal with this subject (6,9,11,24,68).

Perhaps the greatest single reason for the widening interest in microbial adherence is the growing recognition that attachment of bacteria to tissues within the host (e.g., epithelial cells, the tooth surface) is a prerequisite for subsequent colonization (2,9,15,22,33,81,85,102,108,115, 118) and thus an important factor in disease. The current field of bacterial adherence extends far beyond the medical implications. Bacteria adhere to a wide variety of surfaces in the open environment (9,28,29,68,109,121). Bacterial attachment to submerged surfaces in aquatic environments can lead to 'biofouling', a major industrial and economic problem (21). Bacterial adherence, however, is not always a nuisance. Adherence plays a role in beneficiary relationships between host and microorganisms, such as interactions between nitrogen-fixing bacteria and legume root hairs (62,66), and the establishment of normal flora in plants, humans and animals (60-62,78,81,100). The adherent characteristics of microorganisms may be extensively exploited in the future in industrial processes requiring immobilization of microbial cells (31). In some cases, the biological advantages of bacteria which possess adherent properties are clear. The tendency of organic molecules to become concentrated at interfaces, such as submerged solid surfaces (123), or the water:air interface (30,64,68) encourages growth of microorganisms at these

surfaces. Moreover, the ability of microorganisms to adhere may prevent them from being washed from their niche to other less hospitable surroundings (for example, the removal of microbes from the oral cavity to the digestive tract). Adherence plays a fundamental role in the growth physiology of various species. For example Caulobacter cells normally divide only after they attach to solid surfaces (101). Specific cell:cell interactions pervade the entire life cycle of the myxobacteria (34). The ability of a bacterial cell to adhere, however, is not always to its advantage: bacterial attachment is also the first step in phagocytic engulfment (23,113).

While a large number of phenomena involving bacterial adherence have been demonstrated, relatively little is known about the nature of the cell-surface components involved. In some cases, observable exocellular structures have been cited as participants in adherence phenomena. These include stalks and holdfasts (68), polysaccharide layers (22) and fimbriae, or pili (33,35,85). Bacterial fimbriae, defined by Duguid (33) as the "non-flagellar filamentous appendages of bacteria", have been implicated in a variety of adherence phenomena, including attachment to sulfur granules (121), adherence to mucosal cells (33,81,85,118), and interactions among the cells themselves, i.e., pellicle formation (33). The growth of bacteria on insoluble nutrients is often associated with adherence. In those cases in which the carbon or energy source is a water-insoluble material, such as cellulose, or chitin, direct contact between cells and substrate can facilitate growth (60,107), but adherence is not (at least, theoretically) an absolute requirement since extracellular enzymes can degrade these polymers into water-soluble products. However, growth of microorganisms on hydrocarbons presents a special problem since not only are hydrocarbons immiscible with water, but also their breakdown cannot occur extracellularly. The first step in aromatic (40) or aliphatic (88) hydrocarbon degradation is introduction of molecular oxygen into the

molecules by cell-associated enzymes (37). In addition to the interesting theoretical considerations involving adherence, uptake and assimilation of hydrocarbons by microorganisms (53), microbial growth on hydrocarbons has important ecological and industrial implications, particularly in the possible use of microorganisms, or microbial products, in combatting oil pollution problems (41). Two general types of hydrocarbon-cell interactions have been postulated in the literature, depending upon the state and size of the oil droplets relative to the size of the microbial cells (55): adherence of cells to large oil droplets, sometimes termed 'direct contact' (14,70), and pseudosolubilization involving the cellular assimilation of emulsified small hydrocarbon droplets (19). The frequently observed relationship between hydrocarbon:aqueous interfacial area and growth rate can be used to support either hypothesis (75).

While adherence of bacteria and yeast to hydrocarbons during growth has often been observed microscopically (41,55,68,70,75,76), few quantitative methods have been devised in order to examine this phenomenon. Miura et al. (71,72) measured the centrifugal forces necessary to remove adherent yeast cells from tetradecane droplets. These authors were able to demonstrate large differences in the adsorption of three yeast species to the liquid hydrocarbon. The interaction between yeast cells and hydrocarbon has also been studied by measuring the binding of radiolabelled hexadecane droplets to yeast cells (52). Kirschner-Zilber et al. (55) have studied the adherence of a marine pseudomonad to a solid paraffin, n-tetracosane, using a differential filtration technique. Adherence of mammalian cells to liquid hydrocarbon (hexadecane) has also been reported (111). Adherence and growth of microorganisms on hydrocarbons is one example of a wide spectrum of phenomena in which cell-surface hydrophobicity and adherence to hydrophobic surfaces are involved. The degree of hydrophobicity of the outermost microbial cell surface has been cited as a factor in the partitioning of cells at the air:water interface (10,13,16,26,44,56,83,84),

in the adherence of bacteria to non-wettable solid surfaces, such as polystyrene (28,36,39), in the attachment of bacteria to root hairs (66), in phagocytosis (23,113), in certain clumping phenomena (105), and in bacterial adherence to mammalian cells (1,7,86,112,115,118). For example, the ability of Streptococcus pyogenes cells to adhere to buccal epithelial cells has been postulated to occur via a hydrophobic interaction between exposed lipid component of lipoteichoic acid on the bacterial surface and hydrophobic sites on the epithelial cells (7,80,103). In Escherichia coli, correlations have been demonstrated between the presence of certain types of fimbriae-like appendages and bacterial adsorption onto hydrophobic gels (35a,104,115). The observation that rough bacterial strains adhere better to mammalian cells (54,86), and are more liable to phagocytosis than their smooth parents (23,113), has been attributed to the higher cell-surface hydrophobicity of the rough mutants.

The main objectives of the present study were as follows:

(i) To develop appropriate techniques in order to study bacterial adherence to hydrocarbons and other hydrophobic surfaces.

(ii) To gain a better understanding of the nature and extent of bacterial adherence to hydrocarbons and its relationship to other adherence phenomena.

(iii) To examine the role of adherence in bacterial growth on liquid hydrocarbons.

While many bacterial strains were employed in various parts of this study, most of the experiments were performed using Acinetobacter calcoaceticus RAG-1 (ATCC 31012). RAG-1, which has recently been reclassified as an Acinetobacter strain (50;Ophry Pines, unpublished results), was isolated

in 1971 from the seacoast near Tel-Aviv following enrichment for growth on crude oil (90). Members of the genus Acinetobacter are found throughout nature (3,43); they are characterized as gram-negative, non-flaggellar, catalase positive and oxidase negative. Members of the genus are able to metabolize various carbon sources, including unbranched alkanes, but rarely degrade sugars. The use of RAG-1 as the model organism in the majority of this study stemmed from the following observations and data:

(i) Preliminary observations revealed that RAG-1 cells adhere to hydrocarbon droplets during growth on hexadecane in shake flasks;

(ii) RAG-1 produces a potent extracellular emulsifying agent, emulsan, whose physical and chemical properties have been extensively investigated (8,92,99,124,125), thus enabling a direct comparison of the roles of adherence and emulsification in growth on hydrocarbon.

(iii) Various mutants of RAG-1 possessing altered cell-surface characteristics have been isolated in this laboratory, enabling a comparison of their adherence and growth characteristics with those of RAG-1.

MATERIALS AND METHODS

2.1 Bacterial strains

2.1.1 Acinetobacter calcoaceticus Strains

Acinetobacter calcoaceticus RAG-1 (ATCC 31012), an oil-degrading bacterium was previously isolated in this laboratory (90). The following derivative strains were used in this study: (i) Strain RAG-92, a lysine auxotroph (4,87) of RAG-1 obtained from a mutagenized culture, was kindly provided by R. Avigad; (ii) Strain AB15, a non-agglutinating RAG-92 mutant, obtained by selection of cells no longer agglutinated by specific antibodies (4), was kindly provided by E.A. Bayer; (iii) Strain 16tlu, a RAG-92 mutant deficient in emulsan synthesis (5), was kindly provided by E.A. Bayer; (iv) Strains MR-481, RV-1 and RV23-4 were isolated during the course of this work; isolation and properties of these strains are presented in the Results section.

A. calcoaceticus BD4 is a heavily encapsulated strain (50,51) , kindly provided by K. Bryn. Strain BD413 (trp E27) is a minicapsulated derivative of BD4, kindly provided by E. Juni. All other A. calcoaceticus strains used in this study were kindly provided by K. Bryn (17): B5W str 71 (ATCC 15149); B5W (ATCC 17903); ATCC 17906, CDC 7827 (ATCC 17959); ATCC 17977; ATCC 17985; strain '8' (Bacterium anitratum 8, State Serum Institute, Copenhagen, Denmark).

2.1.2 Other Bacterial Strains

Proteus mirabilis smooth strain S1959 and rough derivatives R45 and R51 (98) were obtained from S. Rottem. Photobacterium strains were kindly

provided by S. Ulitzur. Serratia liquefaciens was kindly provided by B. Sneh. The source of all other strains is presented in Table I, in the Results section.

2.1.3 Culture maintenance

Streptococcus pyogenes M-5 was maintained on brain heart infusion agar plates. All other strains were maintained on nutrient agar plates supplemented with 0.5% NaCl. Strains were maintained at 4°C and subcultured every two months.

2.2. Media and Growth Conditions

Starter cultures were obtained by inoculating single colonies from plates into liquid medium. Unless otherwise indicated, erlenmeyer flasks were filled to 20% capacity with medium. Liquid cultures were incubated with shaking (New Brunswick G-53 gyrotory shaker, New Brunswick, N.J.) at 150 revolutions per minute (r.p.m.), at 30°C. Experimental cultures were inoculated from starter cultures grown under the same conditions. The following liquid media were employed: NB- nutrient broth (Difco) supplemented with 0.5% NaCl; BHI- brain heart infusion broth (Difco); CT- 1% casitone and 0.2% $MgSO_4 \cdot 7H_2O$; ethanol medium-PUM buffer (22.2 g $K_2HPO_4 \cdot 3H_2O$; 7.26 KH_2PO_4; 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$, and distilled water to 1000 ml, pH 7.1), supplemented with ethanol (2% v/v unless otherwise specified) and 50 or 100 µg/ml lysine in the case of lysine-requiring RAG-92 strains.

In the experiments which compare adherence of RAG-1 and P. aeruginosa PAS279, (section 3.1.4), the following media were employed for growth on hexadecane in erlenmeyer flasks: Pseudomonas hexadecane medium (shake

flask): 5 g NH_4NO_3, 2.5 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 50 mg threonine and tap water to 1000 ml, supplemented with 0.2% hexadecane; RAG-1 hexadecane medium (shake flask): PUM buffer supplemented with 0.2% hexadecane.

In the growth experiments (sections 3.2.2, 3.6.2, and 3.7.2), different conditions than those stated above were employed. Unless otherwise stated, inocula for these experiments was obtained by growing cells to stationary phase (16-20 h) on brain heart infusion broth. Cells were harvested, washed twice and suspended in PUM buffer, and subsequently inoculated into acid-washed Klett tubes (14 mm diameter) containing 4.2 ml aqueous medium to give an initial turbidity of 5 Klett units. Acetate growth medium consisted of PUM buffer supplemented with 0.2% sodium acetate. Hexadecane growth medium consisted of 0.5 ml of hexadecane layered onto 4.2 ml of PUM buffer. The test tubes were incubated upright in a New Brunswick Gyrotory water bath shaker (model G-76) at 330 rpm at 30°C. Turbidity was monitored directly in the growth tubes by means of a Klett-Summerson colorimeter fitted with a green filter.

Minimal plates were prepared by mixing 500 ml of hot PUM buffer and 500 ml of 4% agar (Bacto-agar, Difco).

2.3 Chemicals and Materials

n-Hexadecane was obtained from Fluka, Switzerland (olefin-free, 99% purity); n-octane was synthetic grade (Merck, West Germany); p-xylene was obtained from Fluka (pure grade).

Slides for visualization of bacterial adherence to epithelial cells were stained with Hemacolor rapid stain (Merck and Co., U.S.A.). Polycarbonate filters (12 µm pore size) for separating epithelial cells from bacteria were obtained from Nucleopore Corp., Pleasanton, Calif.).

Agarose used in crossed immunoelectrophoresis studies was obtained from Pharmacia (Sweden). Glass plates for immunoelectrophoresis (76x51 mm) were obtained from Chance Bros., England. Coomassie brilliant blue R was obtained from Sigma.

Polystyrene disks, used for replica plating, were either 25 mm untreated cover slips specially provided by Lux Scientific Corp., Newbury Park, Calif., or cut from standard bacteriological Petri dishes (NUNC, Sweden, or Miniplast, Ein Shemer, Israel).

Emulsan is a potent extracellular, polymeric emulsifying agent produced by RAG-1 cells (8,87,92,97,124,125). Emulsan employed in the growth experiments (section 3.2.3) and in inhibition of RAG-1 adherence to hexadecane (section 3.4.3) was purified, following ammonium sulphate precipitation (125), by precipitating an aqueous solution of emulsan with cetyltrimethylammonium bromide (BDH), and then dissolving the collected precipitate in 0.5 M Na_2SO_4. The cetyltrimethylammonium bromide was removed by precipitation with KI, followed by extensive dialysis of the supernatant fluid against distilled water. The material was then deproteinized by the phenol method (125) and again dialyzed. This emulsan preparation, kindly provided by Anita Gottlieb, was dissolved in PUM buffer (2mg/ml) and sterilized by autoclaving before it was added in the growth experiments.

All other chemicals and materials were of the highest grade available.

2.4 Adherence to Liquid Hydrocarbons

Bacteria were harvested from growth cultures by centrifugation at 8000 g for 10 min at 4°C, washed twice and suspended in PUM buffer to an initial optical absorbance at 400 nm as indicated. Various volumes of the test

hydrocarbons (n-hexadecane, n-octane, or p-xylene) were added to round-bottom, acid-washed test tubes (10 mm diameter) containing 1.2 ml of washed cell suspension. Following 10 min incubation at 30°C, the test tubes were uniformly vortexed on a Thermolyne Maxi Mix (Sybron Corp., Dubuque, Iowa, U.S.A.) for 120 sec. After allowing 15 min for the phases to separate, the bottom aqueous phase was carefully removed with a Pasteur pipette and transferred to disposable semi-micro cuvettes (C.A. Greiner and Sons, Nurtingen). Light absorbance of the lower aqueous phase was measured at 400 nm in a Gilford model 240 spectrophotometer, 1 cm light path, no filter. Adherence is expressed as the percentage decrease in optical absorbance of the lower aqueous phase following the mixing procedure as compared to that of the cell suspension prior to the mixing procedure.

2.5 Adherence of bacteria to epithelial cells

Epithelial cells were obtained by scraping the buccal mucosa of several healthy donours with wooden sticks (1,81). The cells were washed twice and suspended in phosphate-buffered saline (8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, and distilled water to 1000 ml, pH 7.2) to a concentration of 10^5 cells per ml. An epithelial cell suspension (0.5 ml) was mixed with an equal volume of bacterial suspension (2×10^{10} cells per ml), and the mixtures then were then rotated end over end for 30 min at 37°C (95). These conditions enable optimal adherence. The epithelial cells, bearing adherent bacteria, were separated from the nonadherent bacteria by filtration through polycarbonate filters (12 µm pore size), washed extensively with phosphate-buffered saline and spread onto glass microscope slides. The preparations were air-dried, stained with Hemacolor rapid stain and examined in a bright-field microscope. Adherence was recorded as the percentage of the epithelial cells bearing 100 or more attached bacteria. Clumping of bacteria was not observed in either adherence to hydrocarbons or to epithelial cells.

2.6 Preparation of anti-whole cell RAG-92 antibody

Antiserum was raised against RAG-92 cells by S. Goldman and E.A. Bayer using the following technique. Rabbits were injected intravenously with 10^9 washed cells of RAG-92 cells; the bacteria were harvested from a mid-exponential phase culture of RAG-92 grown on ethanol minimal medium. The rabbits were inoculated in this manner every three days. Three days following the third injection, the rabbits were bled. Subsequent bleedings were performed three days after a booster, consisting of a similar RAG-92 cell preparation. Antibody (IgG fraction) was obtained by precipitation of antiserum in ammonium sulphate at 40% saturation at 0°C; the precipitate was suspended in phosphate-buffered saline to a concentration of ca. 2 mg/ml.

In order to remove from the antiserum preparation antibodies which are common against surface antigens of RAG-92 and the non-agglutinating mutant, AB15, the following method was used (5): an exponential phase culture of AB15 (200 ml) was harvested by centrifugation at 8000 g for 10 min, and the cells washed with 0.15 M NaCl; the washed cell pellet was resuspended in 2 ml of the anti-whole cell antiserum. The mixture was incubated for 2 h at 4°C. After centrifugation at 8000 g for 10 min, the supernatant fluid was subjected to a second adsorption step with a fresh, washed exponential phase culture of AB15. In this manner, only the antibodies which are present on the parent strain (RAG-92) but are absent from the cell surface of mutant AB15 should remain in the adsorbed antiserum preparation.

2.7 Antibody-mediated Agglutination

Antibody-mediated agglutination of RAG-1 cells has been described previously (4). To 0.7 ml cell suspension in semi-micro cuvettes, 0.02 ml

of antibody (diluted ten-fold) were added, and the percentage decrease in absorbance as a function of time (compared to the optical absorbance of a control containing cell suspension with no antibody addition) was followed at 400 nm.

2.8 Crossed Immunoelectrophoresis

Two-dimensional (crossed) immunoelectrophoresis (CIE) of concentrated supernatant fluids of cells subjected to shearing was performed according to a modification of the technique described by Weeke (119). The stock electrophoresis buffer contained 2.06 g sodium barbital, 0.4 g barbituric acid and 0.1 g sodium azide and distilled water to 1000 ml (pH 8.6). The buffer was diluted 1:5 in distilled water, both for use as a running buffer and as a diluent for the 1% agarose. Glass plates were coated with agarose gels (2.0 mm thick).

Supernatant fluids of sheared cells were concentrated five-fold by ultrafiltration, using an Amicon stirred cell fitted with a PM10 membrane filter (Amicon Corp., Lexington Massachusetts). Samples (7 µl) of the concentrated supernatant fluids, containing 30-50 µg/ml protein, were run in the first direction at 25 V cm^{-1} for ca. 1.5 h (bromphenol blue was used as a marker). The second dimension gel contained 10 µl undiluted antibody, or AB15-adsorbed antibody per ml agarose gel and was run at 8 V cm^{-1} for 20 h.

The staining procedure was performed as follows. Following the electrophoretic procedure, gels were pressed to minimal thickness, washed twice with saline (0.85 g NaCl and distilled water to 100 ml), and once in distilled water, dried, and stained for 30 min in 0.02% Coomassie brilliant blue R, dissolved in a solution of 7% (w/v) acetic acid and 50% methanol. Destaining was accomplished using the latter solution.

RESULTS

3.1 Bacterial adherence to liquid hydrocarbons

At the outset of this study, a rapid quantitative technique was devised in order to study bacterial adherence to liquid hydrocarbons (94,96). This assay was based on the initial observation that Acinetobacter calcoaceticus RAG-1 cells, washed and suspended in buffer, could be removed from the aqueous phase upon vigorous mixing ('vortexing') in the presence of hexadecane.

The basic assay for bacterial adherence to hydrocarbons is described in detail in Materials and Methods and illustrated in Figs. 1-3. Cells were harvested,washed twice (to avoid contamination by surfactants) and suspended in 150 mM phosphate buffer, containing small amounts of urea and magnesium sulfate (PUM buffer). This buffer, when supplemented with hexadecane or another suitable carbon and energy source, enables growth of RAG-1 cells. Thus, the assay conditions approximate conditions of adherence of RAG-1 during growth on hexadecane. Turbid, aqueous bacterial suspensions were vortexed under controlled conditions (30°C, 120 sec) in the presence of increasing volumes of liquid hydrocarbon. The test hydrocarbons employed were a short and long-chain n-alkane (n-octane and n-hexadecane, respectively), and an aromatic hydrocarbon (p-xylene). Following phase separation, the turbidity of the lower aqueous phase was measured and compared with that of the original suspension. When the assay was performed with cells which possess little or no affinity towards the test hydrocarbons (e.g. Escherichia coli B), the hydrocarbon droplets rose and coalesced soon after mixing; no significant changes were observed in the turbidity of the aqueous suspension (Fig. 3). However, when adherent RAG-1 cells were similarly tested, a stable 'creamy' upper layer was formed following the mixing procedure (Fig. 1). Microscopic observation of this layer revealed an oil-in-water emulsion consisting of adjacent hydrocarbon droplets in aqueous suspension. Each droplet was

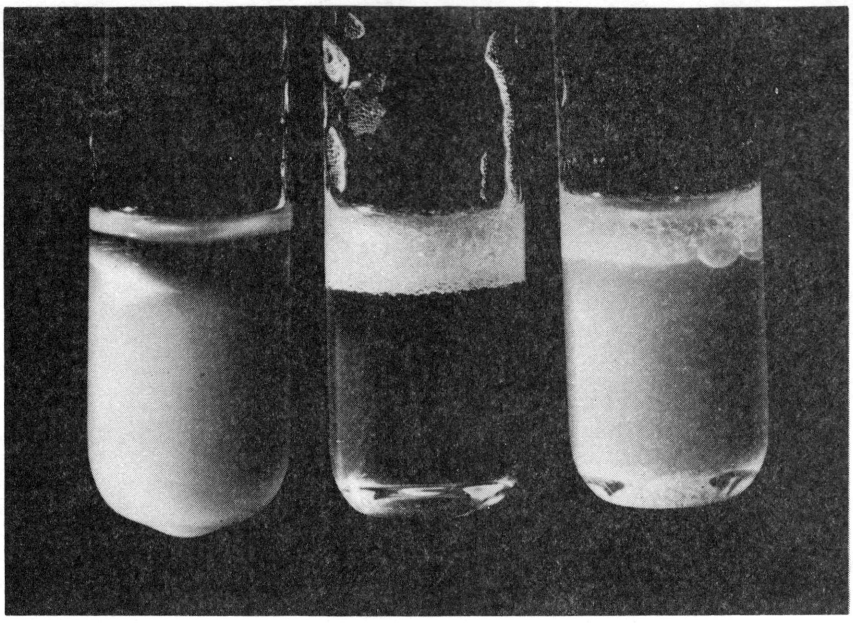

Fig. 1. Adherence of Acinetobacter calcoaceticus RAG-1 to hydrocarbon. Hexadecane was added to an aqueous suspension of A. calcoaceticus RAG-1 cells (left). After mixing for 120 sec and allowing to stand, adherent cells rose with the hydrocarbon, forming a 'creamy' upper layer and a clear aqueous phase (middle). Addition of isopropanol to a final concentration of 5% (v/v) resulted in breakage of the emulsion and release of cells back into the aqueous phase (right).

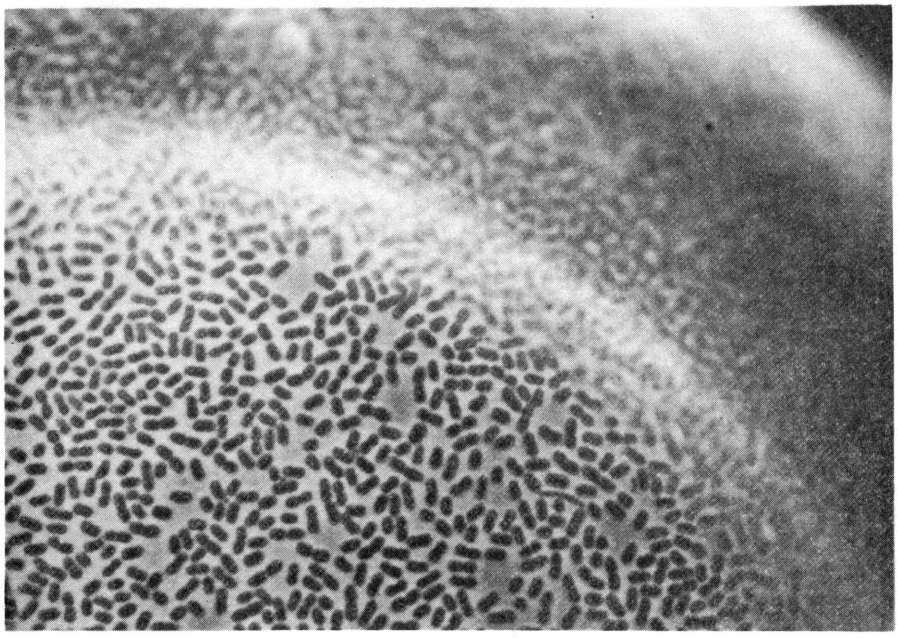

Fig. 2. Phase micrograph showing RAG-1 cells adhering to hexadecane droplet following mixing procedure (x 1350).

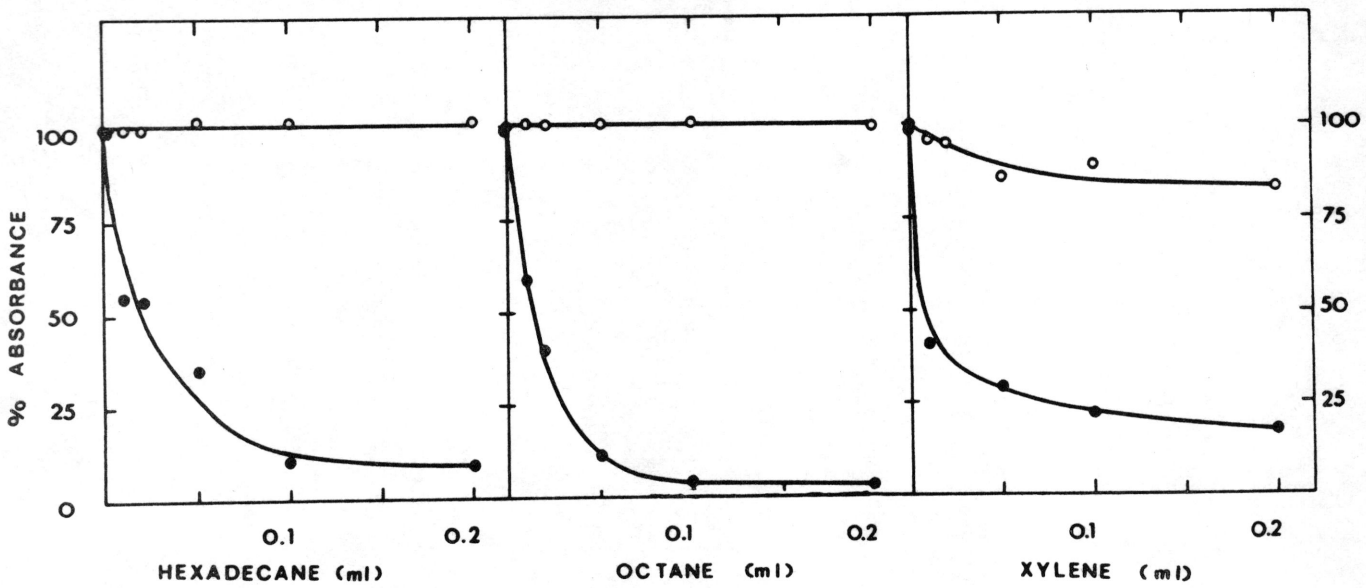

Fig. 3. Bacterial adherence to hydrocarbon. E. coli B (o) and A. calcoaceticus RAG-1 (•) were grown to late exponential phase on nutrient broth. Cells were harvested, washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of 1.4-1.6. Bacterial suspensions (1.2 ml) were vortexed in the presence of different volumes of hexadecane, octane and xylene as described in Materials and Methods. Following phase separation, the turbidity of the lower aqueous phase was measured. Results are expressed as percentage of the initial absorbance of the lower aqueous phase following the mixing procedure as a function of the hydrocarbon volume added.

covered by a layer of bacteria (Fig. 2). Bacteria were not observed within the hydrocarbon phase, but rather adhering at the aqueous:hydrocarbon interface. Cells could be released back into the aqueous phase by addition of surface active agents, such as isopropanol (Fig. 1; see also section 3.4.3). The partitioning of adherent cells at the aqueous:hydrocarbon interface was accompanied by a corresponding decrease in the optical absorbance of the lower aqueous phase (Fig. 1); thus the proportion of adherent cells could be easily determined by comparing the optical density of the aqueous phase following the assay procedure with that of the original bacterial suspension (Fig. 3). The proportion of RAG-1 cells adhering to hydrocarbon increased both as a function of increasing hydrocarbon volume (Fig. 3) and duration of the mixing procedure (Fig.4). Since the mixing conditions employed in the assay may depend somewhat on the specific vortexing apparatus employed, I elected to perform the assay for a fixed duration of time (120 sec) sufficient to allow good attachment of cells (Fig. 4) and to vary the aqueous:hydrocarbon ratio.

3.1.1. Adherence of various bacteria to liquid hydrocarbons

Table I summarizes a large number of experiments in which the adherence of various bacteria to hydrocarbon was tested. The majority of laboratory strains examined exhibited low affinity towards the liquid hydrocarbons. Amongst the bacteria exhibiting high affinity were several hydrocarbon-degrading strains of *Acinetobacter calcoaceticus* (including RAG-1), as well as other bacteria which do not metabolize hydrocarbons: *Serratia marcescens*, *Staphylococcus aureus* and *Streptococcus pyogenes*.

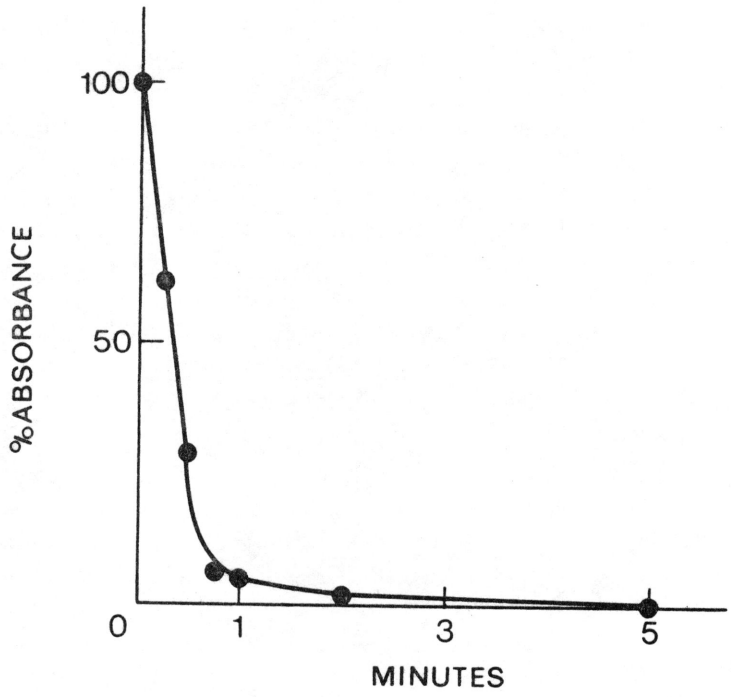

Fig. 4. Adherence to hexadecane as a function of duration of the mixing procedure. RAG-1 cells were grown to early stationary phase on nutrient broth, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.48. To 1.2 ml aqueous cell suspension, 0.2 ml hexadecane were added and the mixtures vortexed for different lengths of time. All other assay conditions were as described in Materials and Methods.

Table I: Adherence of bacteria to hexadecane

Strain	Source	Growth medium[a]	Adherence to hexadecane (%)[b]
Acinetobacter calcoaceticus RAG-1	This laboratory	BHI	97
Bacillus cereus	Local isolate	NB	1
Bacillus subtilis 168	E.Z. Ron	NB	0
Enterobacter aerogenes CDC 659/66	E.Z. Ron	NB	9
Escherichia coli B	E.Z. Ron	NB	0
Escherichia coli K12	E.Z. Ron	NB	2
Escherichia coli J5 (rough)	E.Z. Ron	NB	11
Micrococcus lysodeikticus (ATCC 4698)	I. Friedberg	NB	0
Myxococcus xanthus	This laboratory	CT	35
Proteus mirabilis S1959	S. Rottem	NB	0
Pseudomonas aeruginosa PAS279	J. Shapiro	NB	0
Serratia marcescens	R. Zack	NB	96
Staphlococcus albus	R. Zack	NB	0
Staphlococcus aureus	R. Zack	NB	91
Streptococcus pyogenes M5	I. Ofek	BHI	84

[a] Cells were harvested at the end of the exponential phase, washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of 1.1-1.6.

[b] Adherence to 0.2 ml hexadecane was performed as described in Materials and Methods.

3.1.2 Effect of growth conditions on adherence.

Following development of the basic assay, the effect of different growth conditions on RAG-1 adherence to hydrocarbon was studied. Results are shown in Fig. 5. Age of the cells was of great importance in adherence. While cells grown to stationary phase on complex medium (see also Fig. 11) exhibited optimal adherence properties, and cells harvested in late exponential phase similarly adhered well to hexadecane, cells harvested in early exponential phase following 8-10 generations of growth exhibited extremely low affinity towards hexadecane (Fig. 5C). A similar, but less pronounced increase in adherence with increasing culture age was observed for growth on ethanol (Fig. 5A). Cells grown in shake flasks on hexadecane remained adherent throughout growth (Fig. 5D), in contrast to results reported in another strain of A. calcoaceticus (76).

Similar results were obtained using Serratia marcescens (Fig. 6). Cells harvested at late exponential phase from nutrient broth culture exhibited much greater affinity for the test hydrocarbons than cells harvested during early exponential phase. These results agree with other studies which indicate that appearance of cell-surface components may depend on growth conditions (56,82). The dramatic results obtained with RAG-1 demonstrate the importance of culture conditions on measurements related to bacterial hydrophobicity. This point has sometimes been overlooked (113).

3.1.3 Adherence of Acinetobacter strains.

Eight strains of A. calcoaceticus were compared for adherence to hexadecane, following growth to stationary phase in brain heart infusion broth. Results are shown in Fig. 7. Four strains, including RAG-1, exhibited extremely high affinity for the test hydrocarbon. Two strains (ATCC 17985 and ATCC 17906) were moderately adherent, and two strains (ATCC 17977 and 17903) exhibited extremely low affinity towards hexadecane.

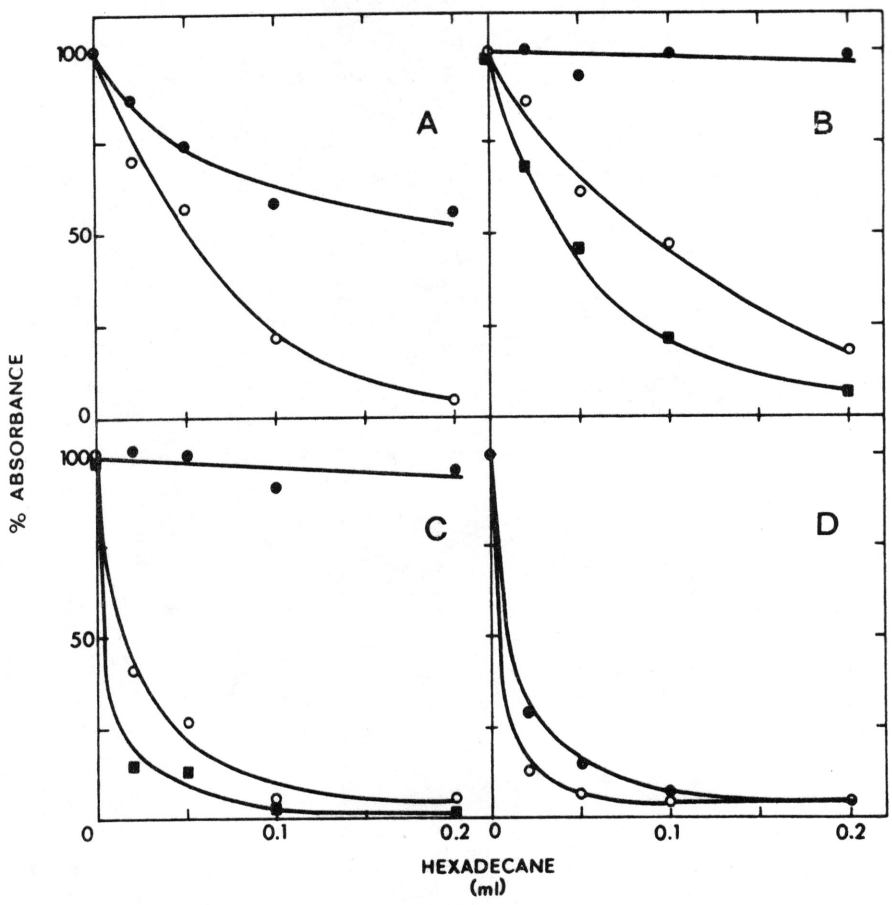

Fig. 5. Effect of culture age and medium composition on adherence of RAG-1 to hexadecane. Following growth as described below, RAG-1 cells were harvested, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.4-1.6. Adherence to hexadecane was performed as described in Materials and Methods. (A) Exponential phase (●) and stationary phase (o) cells grown on 0.5% (v/v) ethanol. (B) Exponential phase (●), late exponential phase (o) and stationary phase (■) cells grown on 0.5% (v/v) ethanol supplemented with 0.05% casamino acids. (C) Exponential phase (●), late exponential phase (o) and stationary phase (■) cells grown in nutrient broth supplemented with 0.5% NaCl. (D) Twenty-four h (●) and fifty-four h (o) cultures grown on 2% (v/v) hexadecane in shake flasks.

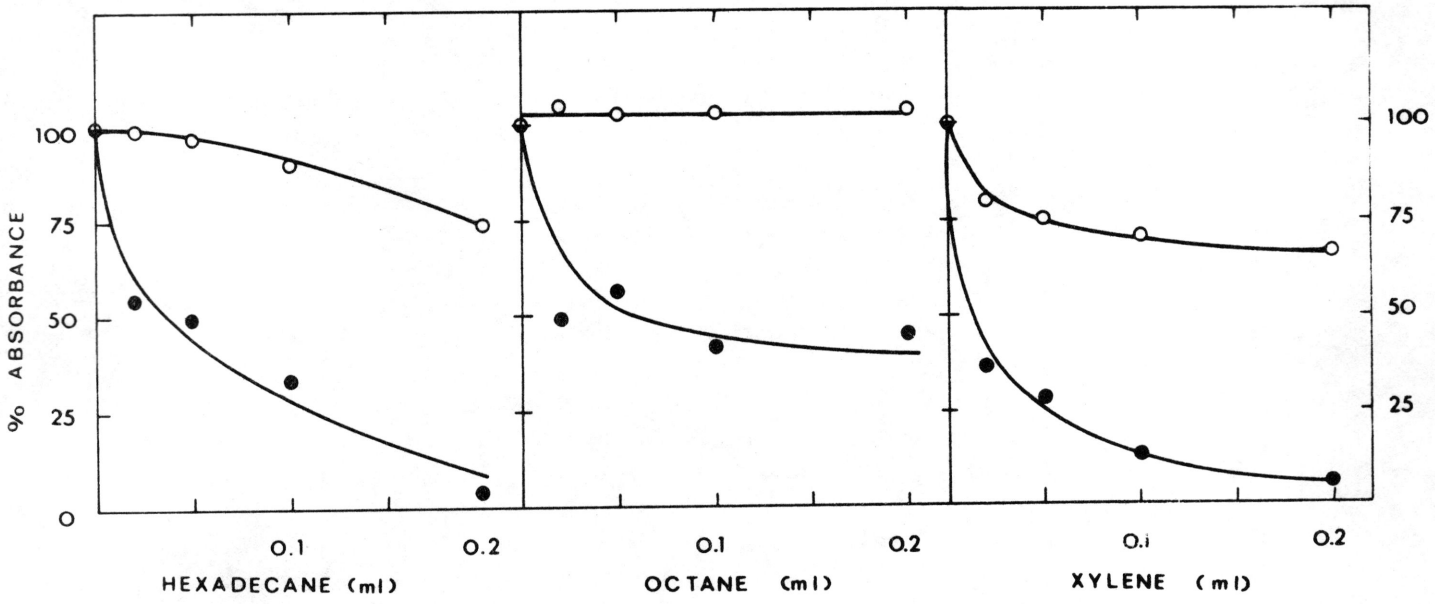

Fig. 6. Adherence of Serratia marcescens to hydrocarbons. Early (o) and late (●) exponential phase S. marcescens cells, grown in nutrient broth, were washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.1-1.2. Adherence to the three test hydrocarbons was performed as described in Materials and Methods.

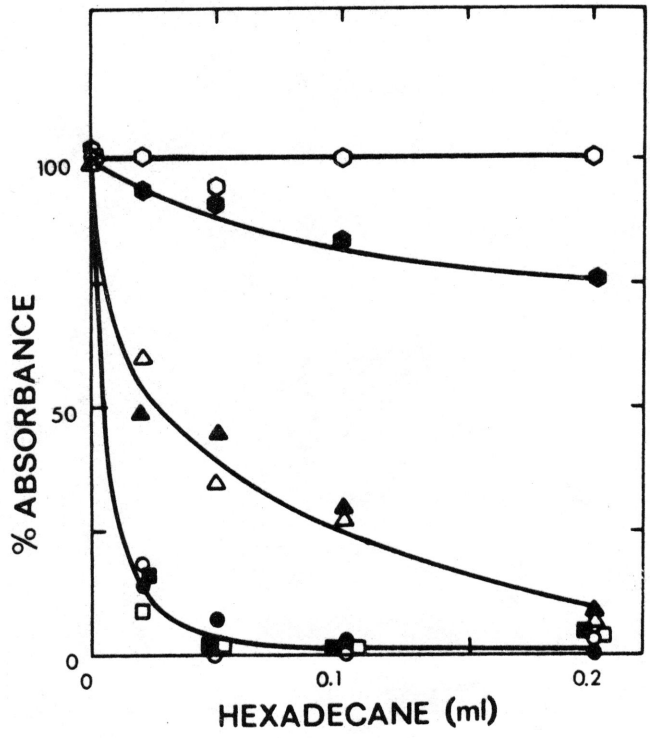

Fig. 7. Adherence of Acinetobacter strains to hexadecane. Cells were grown overnight in brain heart infusion broth, harvested, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.4-1.6. Adherence to hexadecane was performed as described in Materials and Methods. Symbols: (⬡), ATCC 17903; (⬢), ATCC 17977; (△), ATCC 17985; (▲), ATCC 17906; (o), RAG-1 (ATCC 31012); (■), ATCC 15149; (●), ATCC 17959; (◘), Strain '8'.

These results demonstrate that adherence to hydrocarbon varies not only among different bacterial species, but also among strains of a single species.

3.1.4 Comparison of the adherent properties of two hydrocarbon-degrading bacteria.

Adherence of RAG-1 to hydrocarbons was compared with that of Pseudomonas aeruginosa strain PAS 279: both organisms can utilize hexadecane as sole carbon and energy source (46,47). Results are shown in Fig. 8. P. aeruginosa exhibited no significant affinity towards any of the test hydrocarbons when grown to late exponential phase in nutrient broth. Since adherence in this organism may require the presence of hydrocarbon during growth, P. aeruginosa cells were also assayed following growth on hexadecane. The affinity of alkane-grown P. aeruginosa towards hexadecane did increase somewhat, yet it remained nonadherent with respect to the other test hydrocarbons. In contrast, A. calcoaceticus RAG-1 cells were adherent following growth on nutrient broth, and even more adherent following growth on hexadecane. Results demonstrate that avid adherence to hydrocarbon is not a property shared by all hydrocarbon-degrading bacterial strains.

3.1.5. Adherence of rough strains to hydrocarbons.

In a comparison of rough and smooth Proteus mirabilis strains (98), significant differences were observed in their ability to adhere to hydrocarbons (Fig. 9). The smooth parental strain, S 1959, exhibited no affinity towards the test hydrocarbons. Rough strain R 51 was unable to

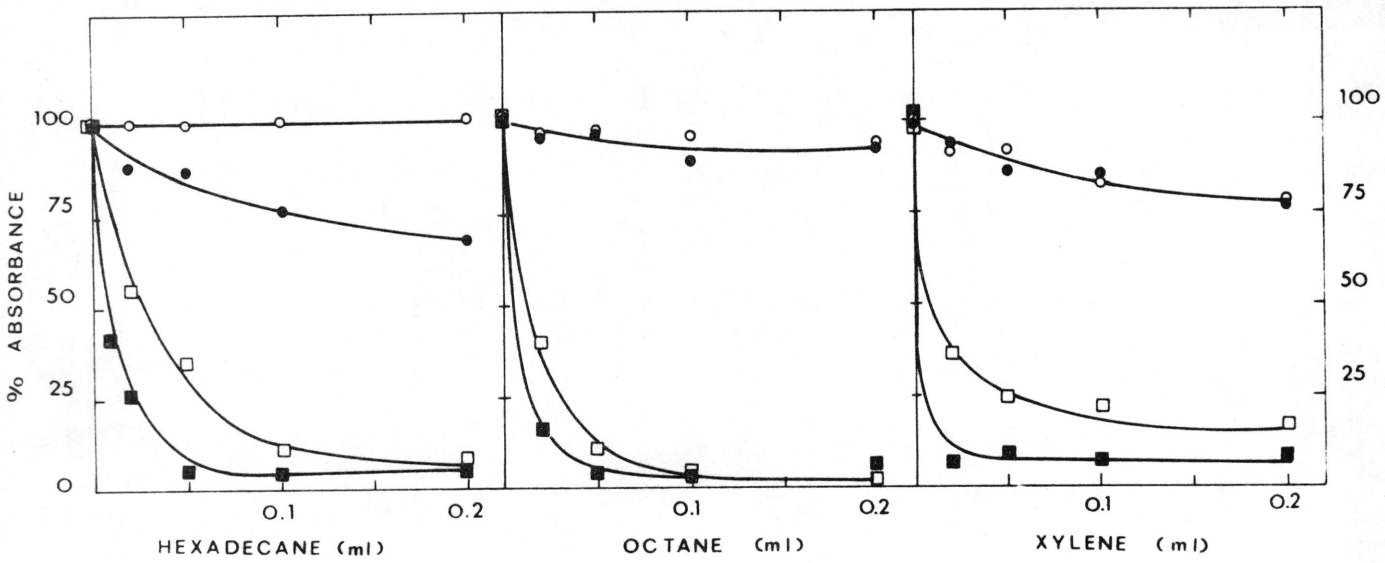

Fig. 8. Comparison of the adherence of two hydrocarbon-degrading strains, A. calcoaceticus RAG-1 and Pseudomonas aeruginosa PAS279. The cells were compared for adherence following growth to late exponential phase on nutrient broth, as well as following 48 hr growth on hexadecane. Cells were washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.20-1.45. Adherence to the three test hydrocarbons was performed as described in Materials and Methods. Symbols: (o), nutrient broth grown PAS279; (●) hexadecane grown PAS279; (□), nutrient broth grown RAG-1; (■), hexadecane grown RAG-1.

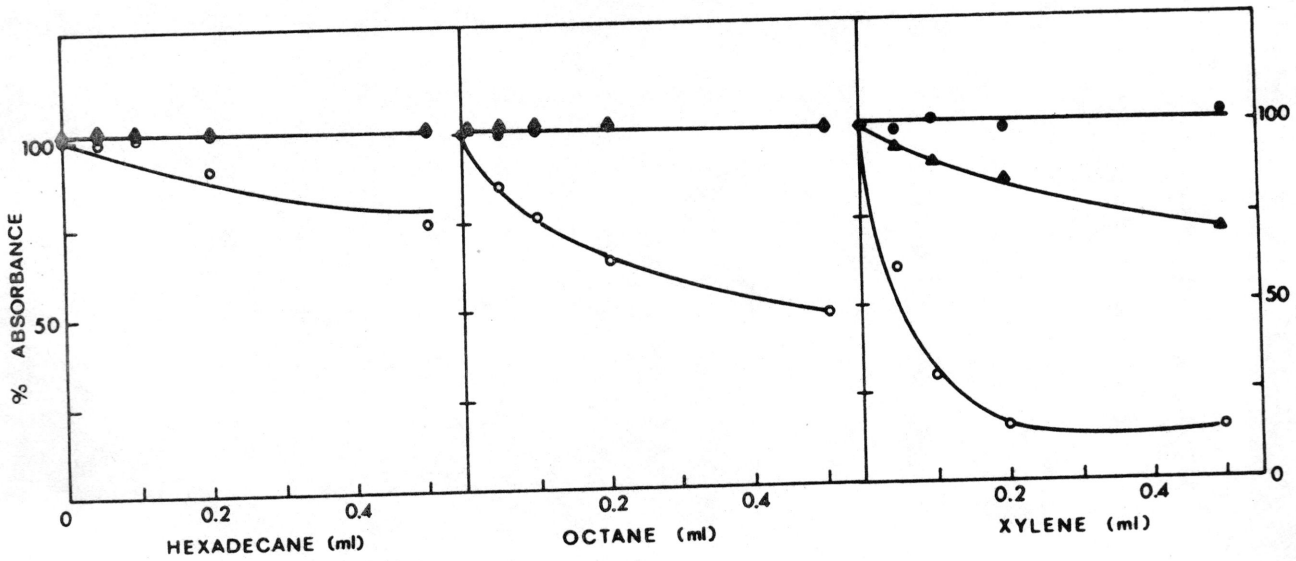

Fig. 9. Adherence of rough and smooth Proteus mirabilis strains to hydrocarbon. P. mirabiis strains were harvested in stationary phase, following 20 hr growth in nutrient broth supplemented with 1.2% NaCl, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.4-1.5. Symbols: (●), smooth strain S1959; (▲), rough strain R51; (o), rough strain R45.

adhere to hexadecane or octane, but did exhibit some affinity towards xylene. Deep rough strain R 45 showed slight affinity for hexadecane and octane and adhered well to xylene. Age of the cultures did not significantly affect the adherence of these cells to hydrocarbon; results similar to those shown in Fig. 9 were observed with cells harvested during exponential phase. Similar, but less pronounced differences were found in a comparison of E. coli K-12 with the rough strain E. coli J-5 (94;Fig. 10).

3.2 Role of Adherence in Growth of A. calcoaceticus RAG-1 on Hexadecane

3.2.1 Isolation of nonadhering mutant MR-481.

In order to study the relationship between adherence and growth of RAG-1 on hexadecane, a spontaneous mutant of RAG-1, deficient in its adherence characteristics, was isolated by the following technique. RAG-1 cells were inoculated into 50 ml of nutrient broth and incubated overnight with shaking. Cells were harvested, washed twice and suspended in PUM buffer to an initial optical absorbance at 400 nm of ca. 1.5. To 5 ml of the cell suspension was added 2 ml of n-octane, and the phases were vortexed for 5 min. After phase separation, the lower aqueous phase was transferred to a second test tube. Octane was again added and the phases were mixed as described above. This extraction process was continued until the cells in the lower aqueous phase were too few to be visible by microscopic examination. This final aqueous phase served as the inoculum for a second nutrient broth culture which was grown to the stationary phase as described above. Cells were again harvested, washed, and extracted with octane; the final aqueous phase was then inoculated into a third nutrient broth culture. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even after several extraction attempts with octane. The final aqueous phase was plated on nutrient agar,

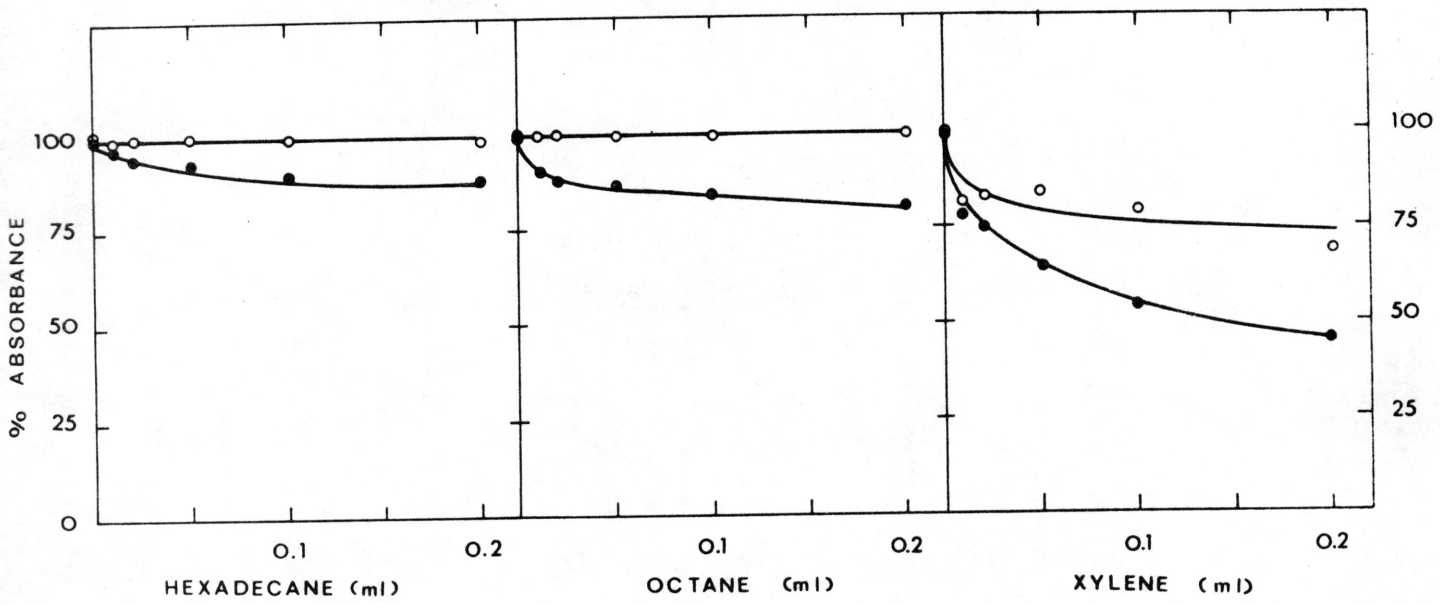

Fig. 10. Adherence of rough and smooth E. coli strains to hydrocarbon. E. coli K-12 (o) and rough strain J-5 (•) were harvested following growth to late exponential phase on nutrient broth, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.4-1.5.

and three individual colony types were isolated and tested for adherence to hydrocarbons. One strain, termed MR-481, exhibited no significant affinity towards the test hydrocarbons, even when grown under conditions for which RAG-1 adherence was optimal (Fig. 11), and was thus chosen for further study.

Mutant MR-481 resembled the wild type in the following characteristics: (A) colonial and cell morphology, (B) sensitivity to bacteriophages (Table VIII) which are specific for A. calcoaceticus RAG-1 strains (87), (C) production of extracellular emulsifying activity during growth on ethanol as sole carbon and energy source (92,125), and (D) growth rate on brain heart infusion broth (38 min at 30°C with aeration).

3.2.2 Growth of RAG-1 and MR-481 on hexadecane

It should be emphasized that in all growth experiments inocula were prepared in the same manner as was described for measuring adherence in Fig. 11. In defined medium with sodium acetate as the soluble carbon and energy source, RAG-1 and MR-481 showed similar growth kinetics (Fig. 12). Following a 2 h lag, both strains grew with a doubling time of 48 min.

Using hexadecane as the sole source of carbon and energy (and under limited dispersion conditions), RAG-1 grew whereas the non-adherent mutant MR-481 failed to grow for at least 54 h (Fig. 13). Growth of RAG-1 was accompanied by breakage of the upper hydrocarbon layer into droplets. Microscopic examination of the upper phase indicated that these droplets were covered by patches of adhering cells. Hydrocarbon droplets were not observed in the aqueous phase under these conditions; thus the observed turbidity of the lower aqueous phase was due to unbound cells. The values presented for growth of RAG-1 on hexadecane should be considered minimal since bacteria attached to the hexadecane droplets were not measured. In the corresponding tubes inoculated with mutant MR-481, neither growth nor substantial breakage of the hexadecane layer into droplets was observed.

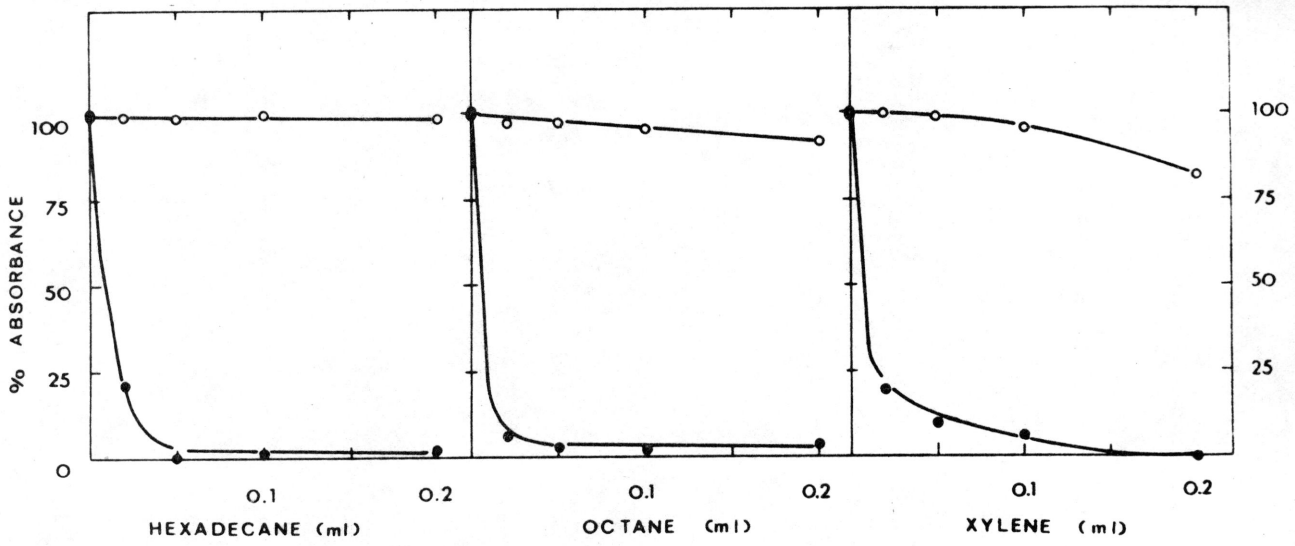

Fig. 11. Adherence of RAG-1 and MR-481 to hydrocarbons. Stationary phase cells were harvested from brain heart infusion broth, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.45-1.50. Adherence to the three test hydrocarbons was performed as described in Materials and Methods. Symbols: (●) RAG-1; (o) MR-481.

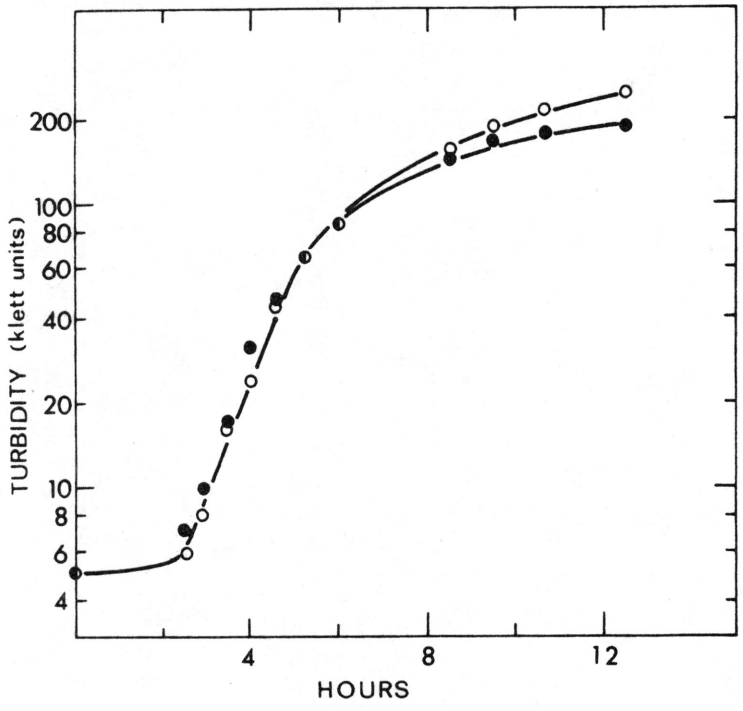

Fig. 12. Growth of RAG-1 and MR-481 in acetate medium. Stationary phase cells were harvested from brain heart infusion broth, washed twice, and suspended in PUM buffer supplemented with 0.2% sodium acetate. RAG-1 (●) and MR-481 (o) cells were inoculated into 4.2 ml of acetate medium in Klett tubes to an initial turbidity of 5 Klett units.

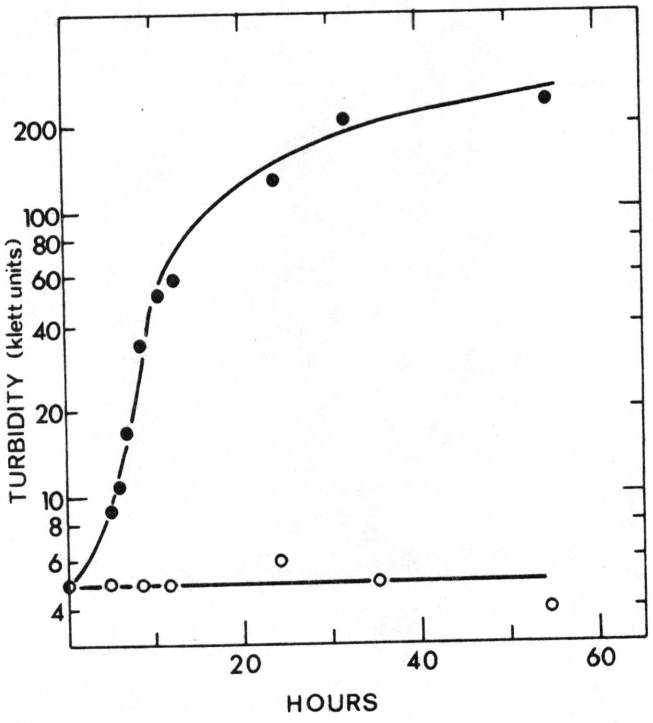

Fig. 13. Growth of RAG-1 and MR-481 in hexadecane medium. Klett tubes containing 4.2 ml of PUM buffer were inoculated with either RAG-1 (●) or MR-481 (o) to an initial turbidity of 5 Klett units. The tubes were overlaid with 0.5 ml of hexadecane and incubated as described in Materials and Methods.

3.2.3 Effect of emulsan on the growth of MR-481 on hexadecane.

A. calcoaceticus RAG-1 produces a potent extracellular emulsifying agent, referred to as emulsan (8,92,124,125). It was of interest to examine the effect of a highly purified preparation of emulsan (see Materials and Methods) on the growth on hexadecane of RAG-1 and the nonadherent mutant MR-481 (Fig. 14). In the absence of hexadecane, emulsan did not serve as a carbon and energy source for either RAG-1 or MR-481. However, emulsan did enable growth of MR-481 on hexadecane. Growth of MR-481 in the presence of both emulsan and hexadecane began after about 6 h, whereas neither component alone supported growth for 54 h. At the weight ratio of hexadecane:emulsan used, 1,925:1, the hexadecane droplets formed were too large to disperse into the aqueous phase and thus did not interfere with turbidity measurements. Emulsan had no significant effect on the growth of RAG-1 on hexadecane under these conditions.

Growth of MR-481 on hexadecane was also induced if the emulsan was added after several hours of incubation (Fig. 15). In each case of emulsan addition, the resultant breakage of the hexadecane layer into droplets was followed by subsequent growth of the mutant cells. The time required for the commencement of growth following emulsan addition appeared to increase with the length of incubation prior to addition of the bioemulsifier.

Growth of MR-481 on hexadecane in the presence of emulsan was not the result of genotypic or phenotypic reversion of MR-481 from nonadherent to adherent cells. As shown in Table II, there was no significant increase in the adherence of MR-481 grown on hexadecane in the presence of emulsan as compared to the initial inoculum grown in rich medium (Fig. 11).

RAG-1 cells which were harvested and washed following growth on hexadecane both in the presence and absence of emulsan, exhibited high affinity towards hexadecane (Table II). Thus, the appearance of free RAG-1 cells in the lower phase during growth on hexadecane did not result from a loss in their adherent properties.

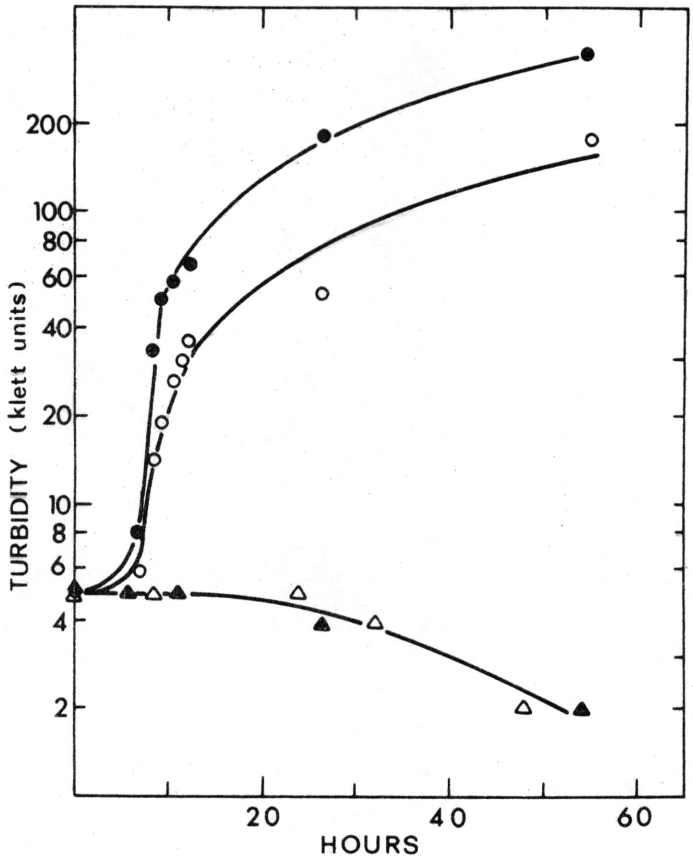

Fig. 14. Effect of emulsan on the growth of MR-481 on hexadecane. The preparation of inocula and incubation conditions were as described in the legend to Fig. 13. RAG-1 (●) and MR-481 (o) were grown on hexadecane medium in the presence of 50 µg/ml emulsan. RAG-1 (▲) and MR-481 (Δ) controls were grown in PUM buffer supplemented with 50 µg/ml emulsan in the absence of hexadecane.

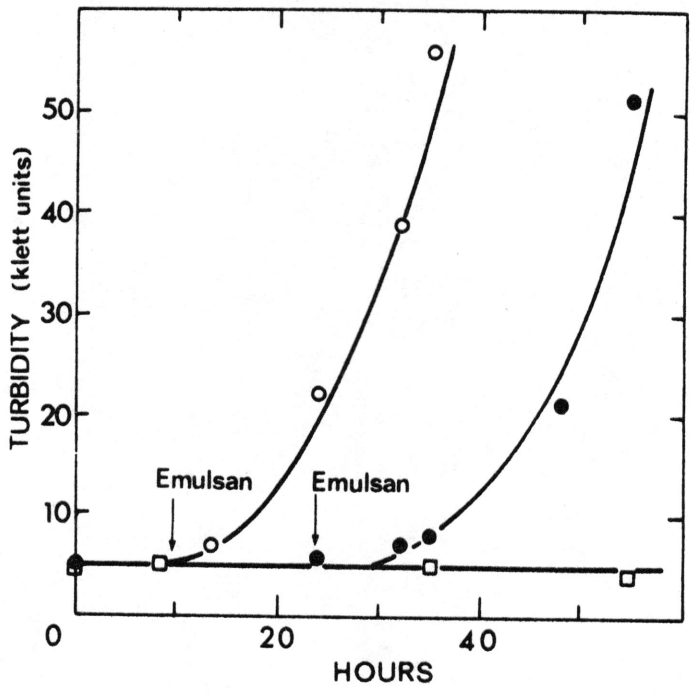

Fig. 15. Induction of growth of MR-481 on hexadecane by emulsan addition. The preparation of inocula and incubation conditions were as described in the legend to Fig. 13. Emulsan was added to a final concentration of 50 µg/ml at 10 (o) or 24 (●) hr. Tubes containing no emulsan served as controls (□).

TABLE II ADHERENCE OF RAG-1 AND MR-481 FOLLOWING GROWTH ON HEXADECANE

Strain	Turbidity (A_{400})[a]		% Adherence
	Initial	Final	
RAG-1[b]	1.42	0.09	94
RAG-1[c]	1.40	0.05	96
MR-481[c]	1.43	1.36	4.9

[a] Cells were washed twice and resuspended in PUM buffer to initial turbidities as indicated. Hexadecane (0.2 ml) was added to 1.2 ml of cell suspension and the mixture vortexed for 120 sec. Following phase separation, the turbidity of the aqueous phase was measured.

[b] RAG-1 cells were harvested following 54 h growth on hexadecane medium, washed twice and suspended in PUM buffer.

[c] RAG-1 and MR-481 were harvested following 54 h growth on hexadecane medium in the presence of 50 µg/ml emulsan, washed twice and resuspended in PUM buffer.

3.3 Adherence of RAG-1 to Other Hydrophobic Surfaces

The ability of RAG-1 to adhere to hydrocarbons which it is unable to utilize, such as octane and xylene (Fig. 11), suggested that RAG-1 adherence to hydrocarbon is mediated by general hydrophobic interactions, rather than by a specific recognition mechanism of metabolizable substrate. It was therefore of interest to see whether RAG-1 adheres similarly to other hydrophobic surfaces.

3.3.1 Adherence to epithelial cells

Adherence of bacteria to epithelial cells, scraped directly from the buccal mucosa, affords a simple *in vitro* model for studying bacterial attachment to host tissue (1,7). Ofek and Beachey have recently proposed a model for adherence of oral streptococci to epithelial cells, stating that adherence occurs primarily via hydrophobic interactions between bacteria and host tissue (7,80,83).

Experiments were devised in order to answer the following questions: (i) Is RAG-1 able to adhere to buccal epithelial cells, despite its non-pathogenic nature? (ii) Can a relationship between RAG-1 adherence to hydrocarbon and to epithelial cells be demonstrated? (iii) Do oral streptococci which adhere to epithelial cells also adhere to hydrocarbon? The avid adherence of RAG-1 cells to human buccal epithelial cells is summarized in Table III. RAG-1 adherence to epithelial cells was closely related to the ability of RAG-1 to adhere to hexadecane. While RAG-1 cells harvested in late exponential phase adhered well to hexadecane and epithelial cells, early exponential phase RAG-1 cells adhered poorly to both surfaces. Adherence to both hexadecane and to epithelial cells was highest when RAG-1 cells were grown to stationary

Table III Adherence of RAG-1 and MR-481 to Buccal Epithelial Cells and to Hexadecane

Strain	Growth medium	Growth phase	% epithelial cells covered with 100 or more bound bacteria[a]	% bacteria adhering to 0.2 ml hexadecane[b]
RAG-1	nutrient broth	early exponential	7	11
RAG-1	nutrient broth	late exponential	43	94
RAG-1	brain heart infusion	stationary	66	100
MR-481	brain heart infusion	stationary	3	2.6

[a] Cells were washed twice and suspended in phosphate-buffered saline; adherence to epithelial cells was determined as described in Materials and Methods.

[b] Cells were washed twice and suspended in PUM buffer. Adherence to 0.2 ml hexadecane was performed as described in Materials and Methods.

phase in complex medium. Mutant MR-481, grown under identical conditions failed to adhere significantly to either surface. RAG-1 adherence to epithelial cells was not affected by addition of various monosaccharides (81,95).

Streptococcus pyogenes M-5, a pathogen and colonizer of the oral mucosa was similarly able to adhere to hydrocarbon. Adherence of strain M-5 to the test hydrocarbons is compared with that of pathogenic E. coli strain 346 (102) in Fig. 16. The adherence of S. pyogenes M-5 to hydrocarbons correlates with previous data on cell surface hydrophobicity of S. pyogenes strains (45,112), and adherence of S. pyogenes to epithelial cells via hydrophobic interactions (1,7,80). While MS^+ (mannose sensitive) strains have been reported to exhibit hydrophobic characteristics (49), MS^+ strain 346 (102) did not adhere to the test hydrocarbons when grown under conditions which promote mannose-sensitive binding (Alex Perry, personal communication).

3.3.2 Adherence to polystyrene: a replica method of screening for bacterial hydrophobicity

RAG-1 cells were observed to adhere to polystyrene, a smooth hydrophobic plastic surface, under various conditions. In preliminary experiments, microscopic observation revealed that cells became firmly bound upon coming into contact with the polystyrene surface. Adherent cells were not removed by repeated washings with PUM buffer or tap water. It was subsequently observed that cells from RAG-1 colonies grown on solid medium also adhered to the polystyrene surface upon contact. These observations led to the development of a simple technique for the screening of colonies of adherent cells, based on bacterial adherence to polystyrene.

In this technique, a flat polystyrene disk was pressed onto the surface of an agar plate containing the colonies to be screened (93). The replica of

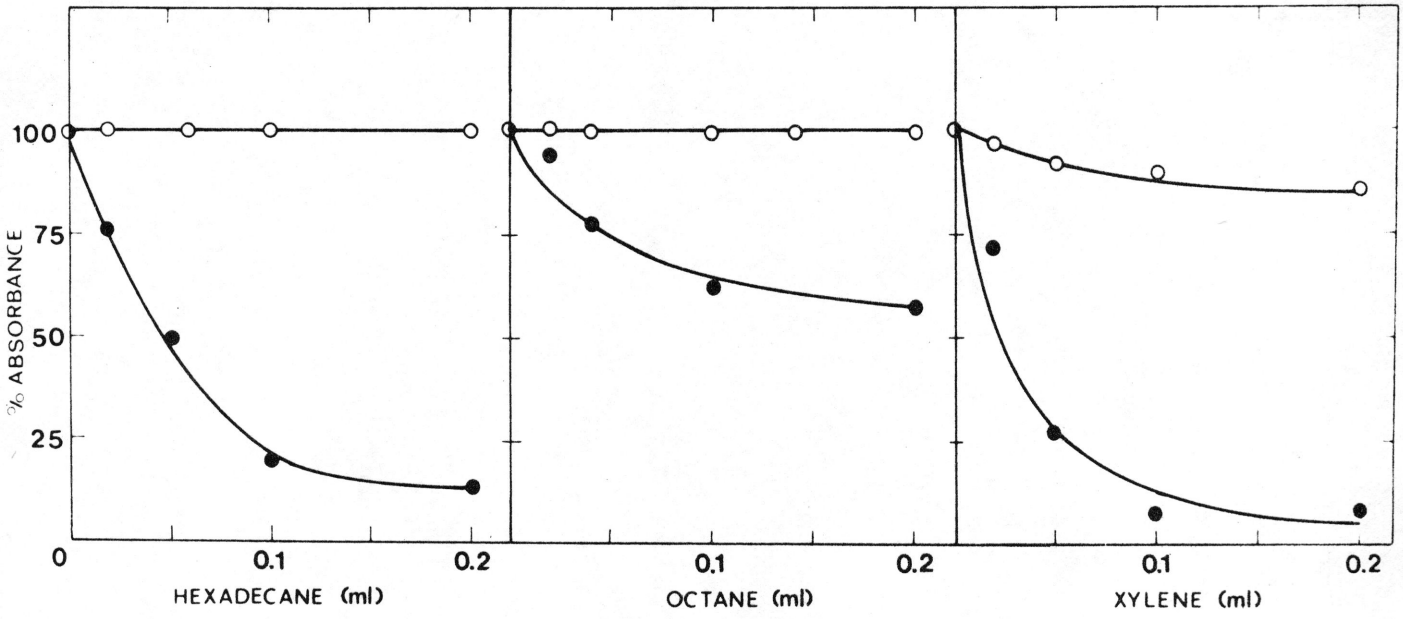

Fig. 16. Comparison of the adherence of two pathogenic strains to hydrocarbon. Streptococcus pyogenes M-5 (H type 5) (●), and Escherichia coli strain 346 (o), were grown at 37°C in brain heart infusion broth under static conditions for 48 hr. Cells were harvested, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 1.4-1.6. Adherence to the test hydrocarbons was performed as described in Materials and Methods. This experiment was performed together with Alex Perry of the Human Microbiology Department, Sackler School of Medicine, Tel-Aviv University.

the colonies obtained on the polystyrene surface was then washed for two min under a vigorous stream of tap water to remove all cells which were not firmly bound. At this stage, translucent areas corresponding to colonies of adherent cells could be observed on the plastic surface; microscopic observation of these spots revealed a dense layer of attached cells. To facilitate visualization and comparison with the original colonies, the replica was fixed by dipping in methanol and then stained with gentian violet.

Results obtained by this technique are shown in Figs. 17-19. In Fig. 17, colonies of RAG-1 and mutant MR-481 were compared. Mutant MR-481 cells were completely removed from the polystyrene replica in the washing procedure, whereas a clear replica of RAG-1 colonies was obtained. Mixtures of RAG-1 and MR-481 colonies which were not distinguishable by colony morphology could be easily resolved by this method (Fig. 18).

Most laboratory strains tested did not adhere to polystyrene and were removed from the disks after the washing procedure. These included colonies of Escherichia coli K-12 DR107, Micrococcus lysodeikticus (ATCC 4698), Myxococcus xanthus FB, Pseudomonas aeruginosa PAS279, A. calcoaceticus (ATCC 17903), and strains of Photobacterium leiognathi, Photobacterium fischeri, and Photobacterium phosphoreum.

Adherence of Serratia marcescens and Serratia liquefaciens strains to polystyrene was found to depend on the age of the colonies. Whereas pink- and red-pigmented colonies were replicated onto the polystyrene, young non-pigmented colonies were not adherent.

Colonies of adherent cells could readily be identified in isolates from natural sources, such as soil samples (Fig. 19).

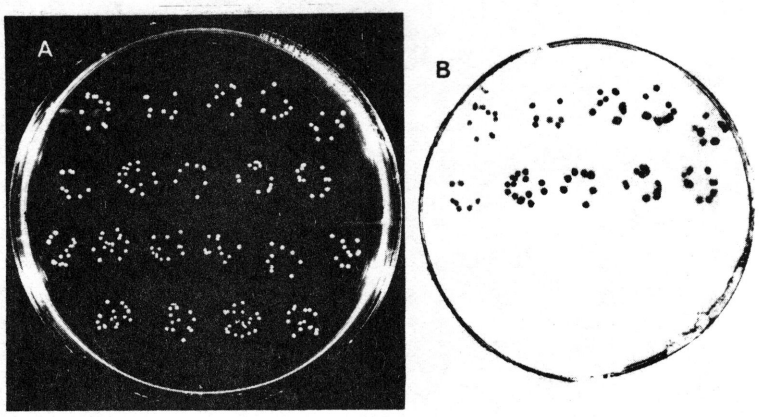

Fig. 17. Replica of RAG-1 and MR-481 colonies. A nutrient agar plate (A) containing colonies of RAG-1 (upper half) and MR-481 (lower half) was replicated onto polystyrene, washed,and MR-481 (lower half) was replicated onto polystyrene, washed and stained as described in Materials and Methods. Only RAG-1 cells were adherent.

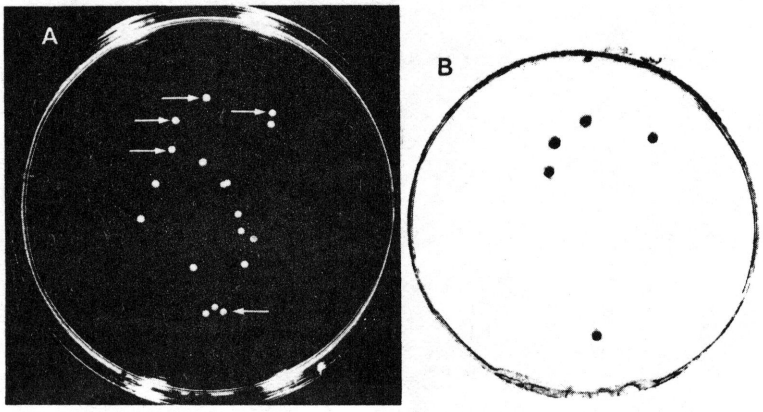

Fig. 18. Replica of mixed RAG-1 and MR-481 colonies. A mixture of MR-481 and RAG-1 cells was plated onto a nutrient agar plate (A). On the polystyrene replica (B), five colonies of adherent cells, indicated by arrows (A), were identified.

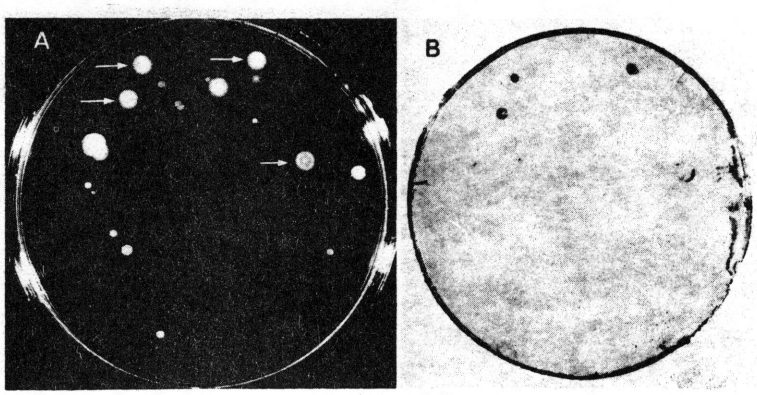

Fig. 19. Replica of colonies from a soil sample. A sample of soil was diluted and plated onto nutrient agar (A) and incubated at 30°C for 48 hr. Several adherent colonies (arrows) were identified on the polystyrene replica (B).

3.4 Inhibition of RAG-1 Adherence to Hydrocarbon and Release of Bound Cells

3.4.1 Effect of shearing on adherence of ethanol-grown RAG-1 cells to hexadecane.

Shearing of cells in a blender-type apparatus is a common method of releasing cell-surface components such as sex pili, fimbriae and flagellae (35). Adherence of ethanol-grown RAG-1 and derivative strains 16-tlu and RAG-92 to hexadecane was considerably reduced following shearing of cells in a Sorvall Omnimixer. Results are shown in Fig. 20 and in Table IV. The decrease in adherence was a function of the duration of shearing. This treatment caused no extensive damage to the cells as observed by phase microscopy.

3.4.2 Effect of sonic oscillation on adherence of RAG-1 cells grown on complex medium.

Sonic oscillation of *E. coli* cells has been reported to decrease their ability to undergo hydrophobic interactions, as measured by retention on hydrophobic gels (82). Adherence of RAG-1 cells grown to stationary phase in brain heart infusion broth was not significantly reduced by the above shearing treatment (decreases in adherence of 5-10% were observed). However, application of sonic oscillation, a more drastic approach to physical disruption of the cell surface, did succeed in reducing adherence of RAG-1 cells grown in complex medium to hexadecane. The effect of sonic oscillation on adherence is shown in Fig. 21. This treatment was far more drastic than the previous shearing technique: two minutes of sonic oscillation of RAG-1 cells under these conditions reduced the turbidity of the cell suspension by a factor of 50%.

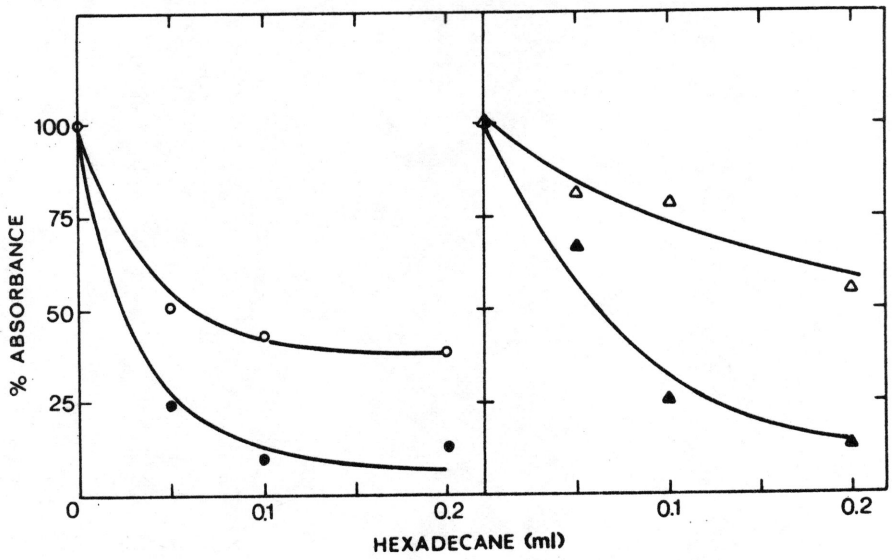

Fig. 20. Effect of shearing on adherence of RAG-1 and RAG-92 to hexadecane. Cells were harvested, following 48 hr growth on 2% (v/v) ethanol medium, washed twice and suspended in PUM buffer to ca. 1000 Klett units. Cells were subjected to five minutes shearing in a Sorvall Omni-mixer at a power of 9, at 0°C. Cells were then harvested and resuspended in PUM buffer to an initial absorbance at 400 nm of 1.35-1.45 and assayed for adherence to hexadecane. Symbols: (o), RAG-1 cells, subjected to shearing; (●) non-sheared RAG-1 cells; (Δ), RAG-92 cells, subjected to shearing; (▲), non-sheared RAG-92 cells.

Table IV. Effect of Shearing on Adherence of Ethanol-grown RAG 16-tlu[a]

Duration of Shearing (min)	Adherence to hexadecane[b]
0	93
1	77
2	64
5	54

[a]RAG 16-tlu, a spontaneous translucent mutant of RAG-92, was harvested following 48 hours growth on 2% (v/v) ethanol medium supplemented with 100 µg/ml lysine. Cells were harvested, washed twice, and suspended in PUM buffer to an absorbance at 400 nm of ca 1.5. Cells were then sheared for different time periods, as described in Fig. 20.

[b]Sheared cells were harvested and suspended in PUM to an initial absorbance at 400 nm of 1.23-1.43 and assayed for adherence to 0.2 ml hexadecane as described in Materials and Methods.

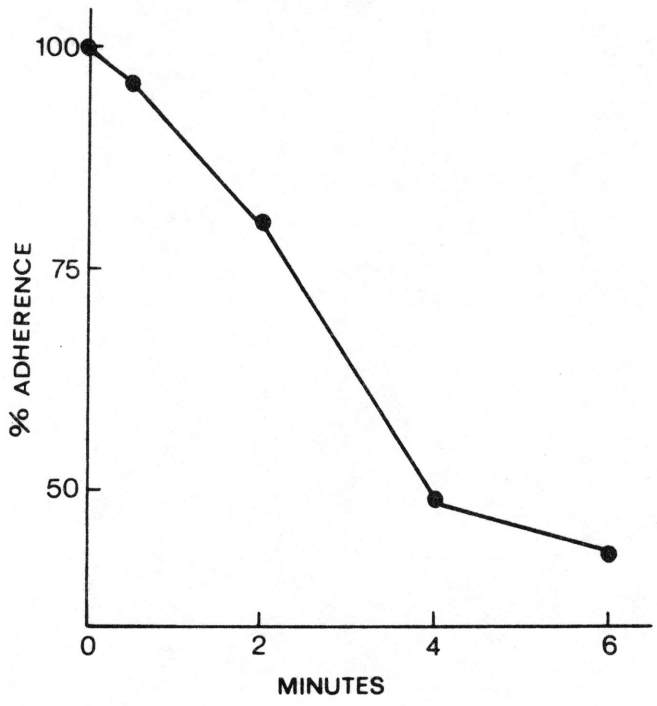

Fig. 21. Effect of sonic oscillation treatment on adherence of RAG-1 cells grown in complex medium. RAG-1 cells were harvested following growth to stationary phase on brain heart infusion broth, washed twice and suspended in distilled water to a concentration of 43 mg/ml dry weight of cells (ca. 15,000 Klett units). Cells were exposed to sonic oscillation at 0°C, for different lengths of time, using a Labsonic 1310 (Braun) sonifier (needle probe), at 70 watts power. Following treatment, cells were harvested and resuspended in PUM buffer to an initial absorbance at 400 nm of 1.20-1.35, and adherence to 0.2 ml hexadecane assayed. Results are expressed as percentage adherence to hexadecane as a function of the duration of the treatment.

3.4.3 Effect of emulsan addition on RAG-1 adherence to hexadecane

Emulsan has a high affinity for the aqueous:hexadecane interface (124). It was of interest to see whether emulsan would (i) compete with RAG-1 cells in partitioning at the interface, and (ii) remove RAG-1 cells which were already bound to the hexadecane. As shown in Table V, addition of emulsan prior to the mixing procedure completely inhibited RAG-1 adherence to hexadecane. Moreover, when emulsan was added following the assay procedure (i.e., after 94% of the cells had become bound to the hexadecane) and the phases were once again mixed, only 27% of the cells remained bound.

3.4.4 Effect of liquid-solid hydrocarbon phase transition on release of bound cells

RAG-1 cells bound to hexadecane could be released by reducing the temperature to values <16°C which caused the hexadecane to solidify. After allowing the mixture to thaw, coalescence of the hexadecane droplets was observed, together with release of cells back into the aqueous phase. (Fig. 22). Microscopic observation of the aqueous phase following coalescence revealed small clumps of cells as well as individual bacteria. From 45-60% of the initial turbidity of the cell suspension could be recovered in this manner. This effect was not a direct result of temperature changes, since cells bound to a hydrocarbon with a low melting point, such as octane (melting point: -56.5°C) were not released under these conditions (Fig. 22).

3.5 Isolation of partial adherence revertants RV-1 and RV23-4

The nonadherent RAG-1 mutant MR-481 was unable to grow on hexadecane in liquid culture under the conditions described in section 3.2.2 for at least

Table V Effect of Emulsan Addition on RAG-1 Adherence to Hexadecane

Treatment	Absorbance (A_{400})	% Adherence
None (control)	0.095	94
Emulsan[b] added before mixing procedure	1.518	0.8
Emulsan[c] added following mixing procedure	1.124	27

[a] RAG-1 cells were harvested following growth to late exponential phase in nutrient broth, washed and suspended in PUM buffer to an initial optical absorbance at 400 nm of 1.53. Adherence to 0.2 ml hexadecane was performed as described in Materials and Methods.

[b] Final concentration, 100 µg/ml

[c] Following the mixing procedure and phase separation, emulsan was added and the phases again vortexed for 120 seconds. Turbidity was measured following phase separation.

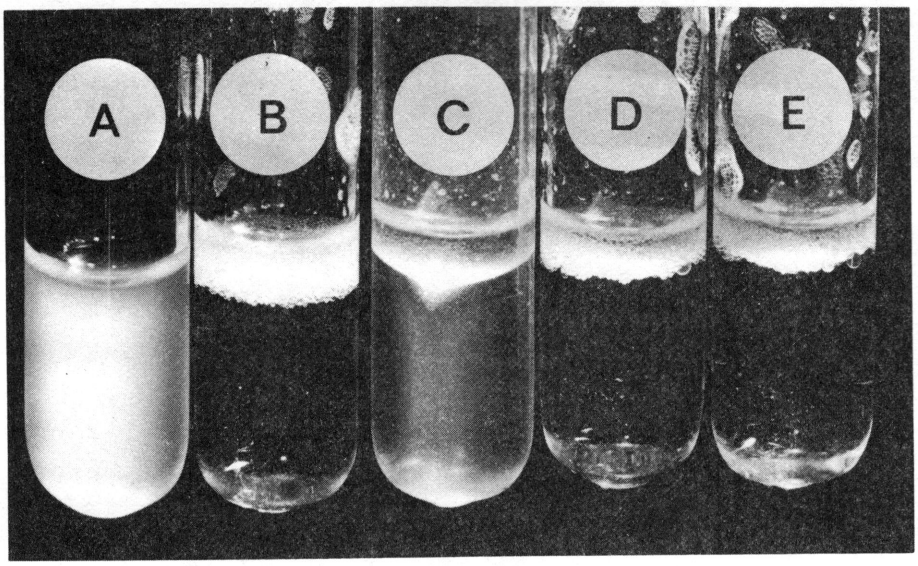

Fig. 22. Release of cells bound to hexadecane due to phase transition of the hydrocarbon. RAG-1 cells, grown to stationary phase on brain heart infusion broth, washed twice and suspended in PUM buffer, were tested for adherence to 0.2 ml hexadecane (tubes B and C), and 0.2 ml octane (tubes D and E) as described in the legend to Fig. 11. Following the adherence assay, test tubes C and E were immersed in a water bath at 12°C for three minutes, then allowed to equilibrate with room temperature (22°C). In the tube containing RAG-1 cells bound to hexadecane (tube C), the hexadecane droplets solidified, and following equilibration at room temperature, melted and coalesced, with concommitant release of RAG-1 cells back into the aqueous phase. No such effect was observed in a similarly treated tube (tube E) containing RAG-1 cells bound to octane. Test tube A contains the washed bacterial suspension, prior to the adherence assay.

54 h (Fig. 12). However, it was subsequently observed that growth did sometimes ensue following additional incubation. It was of interest to see whether this phenomenon was due to (i) contamination, (ii) slow eventual growth of MR-481 cells, or (iii) enrichment of revertants or partial revertants of MR-481 with respect to adherent properties.

In order to study this phenomenon, twenty hexadecane growth tubes (Materials and Methods, and Results section 3.2.2) were inoculated with MR-481 cells (pregrown and washed as in the hexadecane growth experiments, Results section 3.2.2) to an initial turbidity of 10 Klett units. Following 72 h incubation, considerable growth was observed in four of the test tubes (>150 Klett units). These four cultures were plated onto solid medium (nutrient agar) and at least 50 colonies of each culture were replicated onto polystyrene (section 3.3.2). Over 95% of the colonies tested from each of the four tubes adhered to polystyrene. In contrast, of 6000 colonies isolated from the MR-481 inoculum of the same experiment, not one colony adhered to the polystyrene replica. One clone was subsequently isolated from each of the four growth tubes. These four clones were termed RV-1, RV-2, RV-3 and RV-4. In preliminary studies, the four strains appeared very similar in a number of properties (adherence to hexadecane and polystyrene, agglutination by antibodies, production of emulsan following growth on ethanol, and cell morphology, as observed in uranyl acetate-stained electron micrographs). These, and other characteristics are described in the following section (3.6). Because no significant differences were observed in these strains, one clone (RV-1) was arbitrarily chosen for further investigation.

In addition to regaining the ability of wild type colonies to adhere to polystyrene plates (Fig. 27), RV-1 adhered somewhat better to the test hycrocarbons than MR-481 (Fig. 23), but still poorly compared with wild type RAG-1 cells. I was thus interested to see whether additional passages of strain RV-1 on hexadecane would lead to the selection of more adherent cells. To test this hypothesis, RV-1 cells were inoculated into hexadecane

growth cultures (as described in section 3.2.2) and, following growth, were inoculated 1:50 into a fresh hexadecane growth culture. Following subsequent growth, the culture was streaked onto solid medium, and eight colonies were isolated and tested for adherence to hexadecane. One clone, termed RV23-4, did show significantly increased adherence to hydrocarbon.

3.6 Comparison of Properties of RAG-1, MR-481, RV-1 and RV23-4

3.6.1 Adherence to hydrocarbons.

Adherence of RAG-1, non-adherent mutant MR-481, and strains RV-1 and RV23-4 to the three test hydrocarbons following growth on complex medium is shown in Fig. 23.
RV-1 exhibited slightly increased affinity towards hexadecane and xylene with respect to MR-481 (Fig. 23). No significant difference was observed in adherence of these two strains to octane.

RV23-4 adhered with higher affinity to all three test hydrocarbons than RV-1 or MR-481; however, adherence of this strain was still appreciably lower than that observed for wild type RAG-1 cells. Strains RV23-4, also was able to adhere to polystyrene (Fig. 27).

Adherence data for the four strains were also obtained following growth on solid minimal medium supplemented with hexadecane vapours (Table VI). The results again indicate that growth of MR-481 on hexadecane selected for increasingly adherent variant strains.

3.6.2 Growth on hexadecane

In order to test whether the partial adherence revertants RV-1 and RV23-4, which were enriched for by growth on hexadecane in liquid culture, were

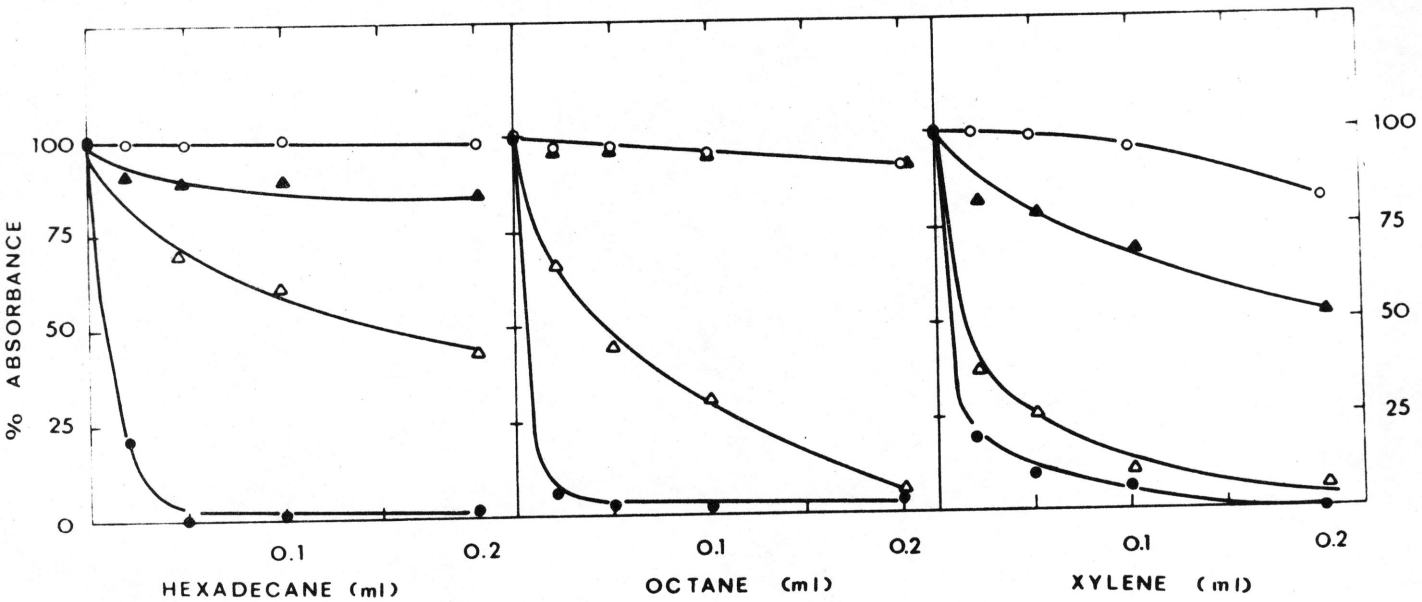

Fig. 23. Adherence of partial RAG-1 revertants RV-1 and RV23-4 to hydrocarbon. Following growth to stationary phase in brain heart infusion broth, cells were washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of 1.46-1.56. Adherence to the three test hydrocarbons of RV-1 (▲) and RV23-4 (Δ) is compared with the adherence of RAG-1 (●) and MR-481 (o).

Table VI Adherence to Hexadecane Following Growth on Hexadecane Vapours[a]

Strain	% Adherence to hexadecane
RAG-1	90
MR-481	4.5
RV-1	16
RV-23-4	55

[a] Cells were grown 72 h on minimal plates supplemented with hexadecane vapours. Cells were scraped from the plate, washed twice and suspended in PUM buffer to an initial absorbance of 1.45 - 1.55. Adherence to 0.05 ml hexadecane was as described in Materials and Methods.

indeed able to grow under these conditions, the growth experiments described in section 3.2.2 were repeated, this time including strains RV-1 and RV23-4 (Fig. 24 and 25). In defined medium with sodium acetate as the substrate, all four strains grew similarly, with a doubling time of 50 min (Fig. 24). Using hexadecane as the sole carbon and energy source (Fig. 25), growth kinetics of RAG-1 and MR-481 were similar to those reported in section 3.2.2. RAG-1 grew, following a short lag, with an apparent doubling time (as measured by the increase in turbidity in the aqueous phase; bound cells were not determined) of approximately one hour, reaching 115 Klett units following 34 h incubation. The nonadherent mutant MR-481 failed to grow on hexadecane under these conditions, as previously described (section 3.2.2). Growth kinetics on hexadecane were similar for RV-1 and RV23-4: following a lag of about
seven hours, growth commenced with an apparent doubling time of approximately two hours. Strain RV23-4 grew to a higher cell density than RV-1, reaching 57 Klett units after 34 h, as opposed to 37 Klett units, obtained for RV-1 cells.

3.6.3 Fimbriation: Electron microscopic examinations

RAG-1 cells possess at least two distinct kinds of fimbriae, or pili, as was observed in electron micrographs of cells stained with uranyl acetate: 'thin' fimbriae, ca. 3.5 nm in diameter, which are generally predominant, and 'thick' fimbriae, approximately 6.5 nm in diameter (Fig. 26A). The thin fimbriae appear be fairly rigid, and vary in length from ca. 200 nm to over one micron; the thick fimbriae appear curled and twisted. Only thin fimbriae were observed on RAG-1 cells grown on minimal ethanol medium (E.A. Bayer, unpublished results). Fimbriation (of both types) was observed to increase with the age of cells grown in nutrient broth. While the surface of early exponential phase cells was almost completely bare (not shown), late exponential and stationary phase cells were covered with numerous fimbriae (Fig. 26A).

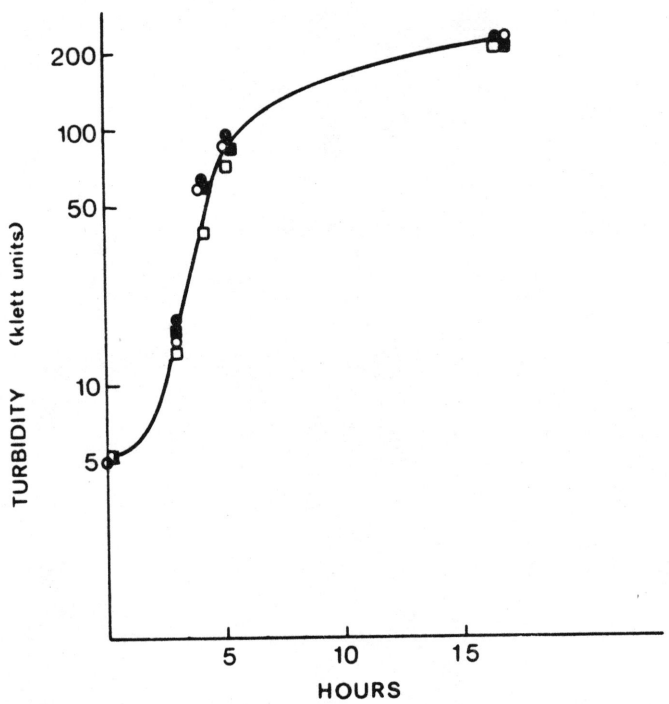

Fig. 24. Growth of RV-1 and RV23-4 in acetate medium. Stationary-phase cells were harvested from brain heart infusion broth, washed twice and suspended in PUM buffer supplemented with 0.2% sodium acetate. RV-1 (□), RV23-4 (■), RAG-1 (●), and MR-481 (o) cells were inoculated into 4.2 ml of acetate medium in Klett tubes to an initial turbidity of 5 Klett units.

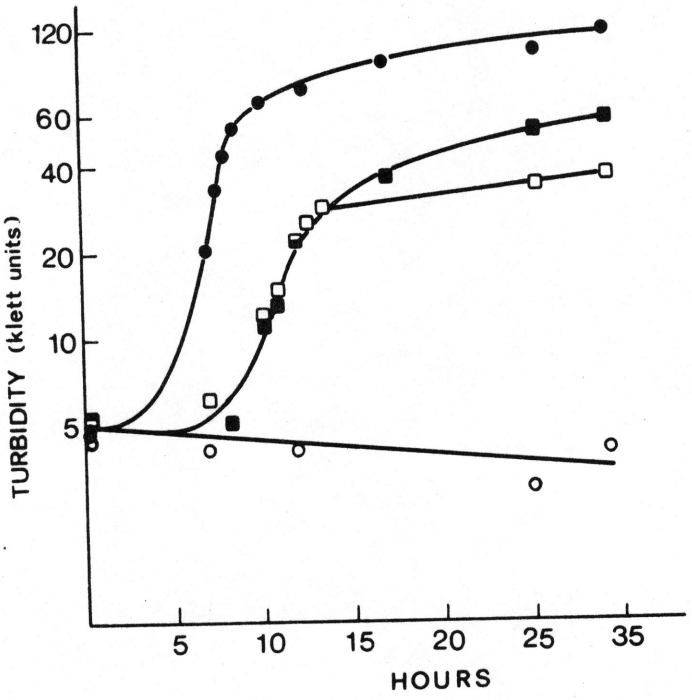

Fig. 25. Growth of RV-1 and RV23-4 in hexadecane medium. Klett tubes containing 4.2 ml of PUM buffer were inoculated with either RV-1 (□), RV23-4 (■), RAG-1 (●), or MR-481 (o) to an initial turbidity of 5 Klett units. The tubes were overlaid with 0.5 ml hexadecane and incubated as described in Materials and Methods.

In contrast to RAG-1, nonadherent MR-481 cells were completely, or almost completely, devoid of thin fimbriae (Fig. 26B). In many electron micrographs of MR-481 cells grown in different media and harvested at various stages of growth no thin fimbriae were observed; however, occasionally, up to five fimbriae resembling those of the wild type cells were observed on MR-481 cells. MR-481 cells grown on complex medium were covered with fimbriae of the thick variety (Fig. 26B).

Electron micrographs of the partial adherence revertants RV-1 and RV23-4 sharply contrasted with those of MR-481. RV-1 (Fig. 26C) and RV23-4 (Fig. 26D) both possessed a multitude of thin fimbriae. A comparison of electron micrographs indicated that the surfaces of both partial revertants were covered with more thin fimbriae and fewer thick fimbriae than the corresponding wild type RAG-1 cells. Both partial revertant strains were also densely fimbriated (thin variety) following 48 hr growth on 2% (v/v) minimal ethanol medium.

The type and extent of fimbriation present in the four strains, following growth on complex medium and on ethanol, is summarized in Table VII.

3.6.4 Phage-typing

Various phages, specific for RAG-1 cells, have been isolated in this laboratory (87; Ophry Pines, unpublished results). One phage, termed $\phi 1$, is specific for certain RAG-1 mutants, while RAG-1 cells themselves are resistant. The results of phage-typing the four strains are summarized in Table VIII. The experiment was performed together with Ophry Pines. Although no differences were observed in the phage specificities of RAG-1 and MR-481, partial revertants RV-1 and RV23-4 each exhibited altered phage patterns with respect to the wild type, as well as with respect to one another.

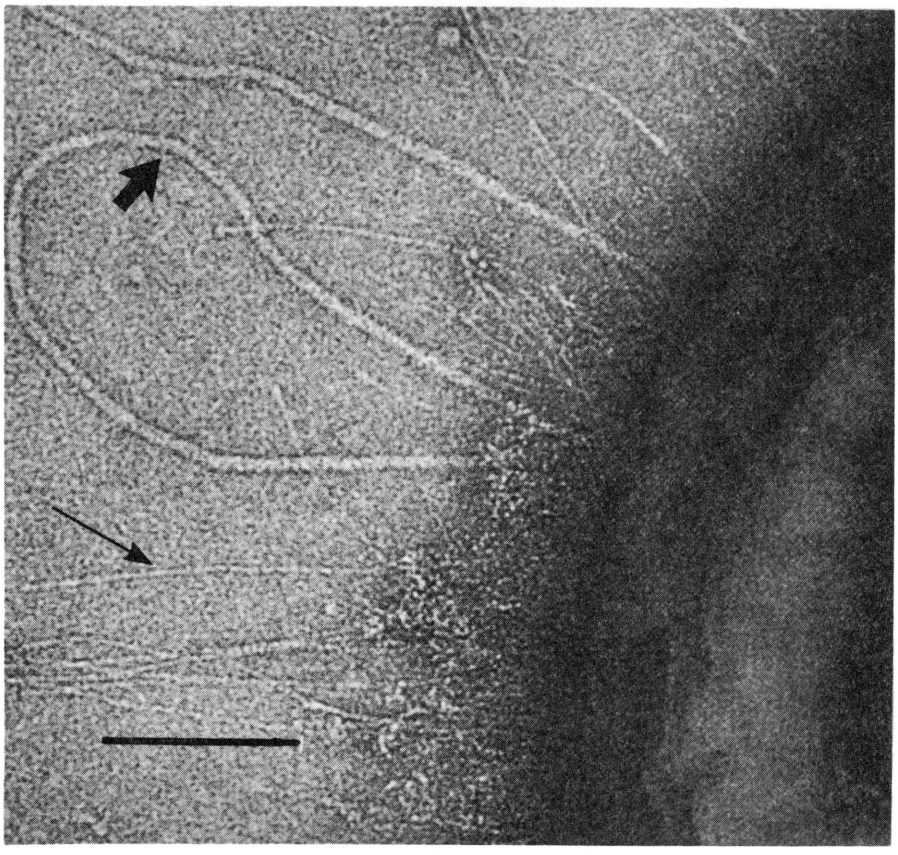

Fig. 26A

Fig. 26. Fimbriation of RAG-1, MR-481, RV-1 and RV23-4. Cells grown to stationary phase in brain heart infusion broth were harvested, washed and suspended in distilled water. Cells were stained with 0.5% uranyl acetate, and observed in the JEOL 100 B electron microscope. A - RAG-1; B - MR-481; C - RV-1; D-RV23-4. Bars represent 0.1 μm. Thin arrows indicate thin fimbriae; thick arrows indicate thick fimbriae. Electron microscopy was performed by Jacob Delarea.

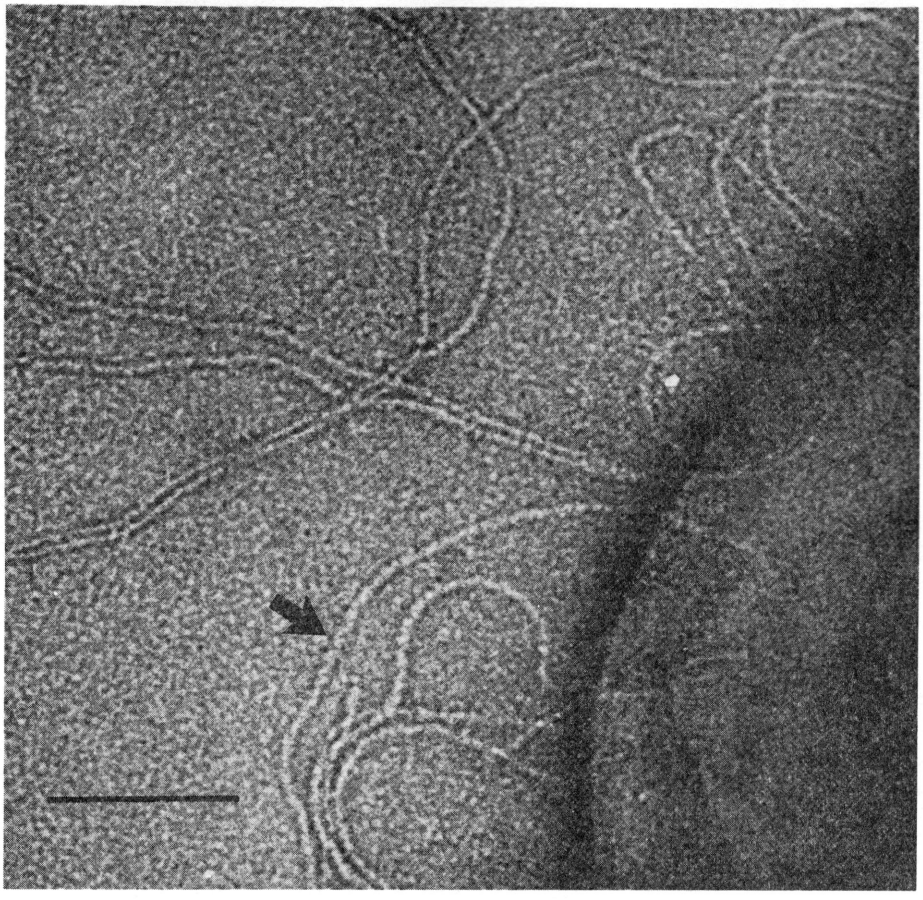

Fig. 26B

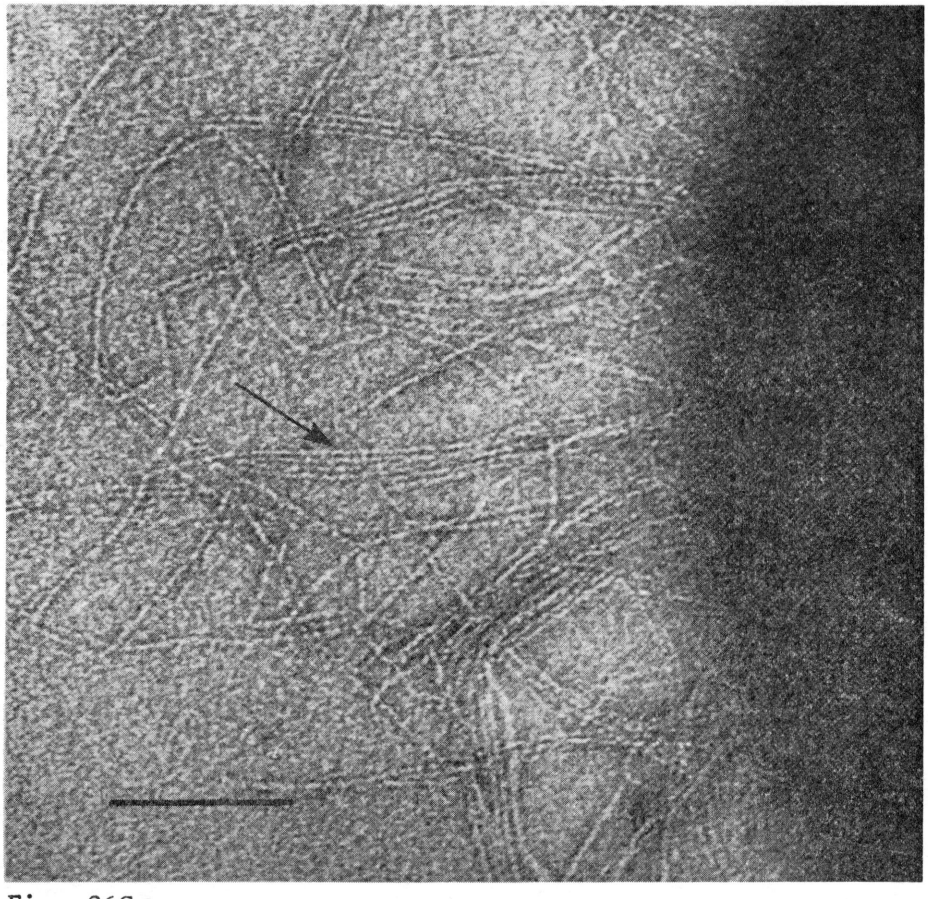

Fig. 26C

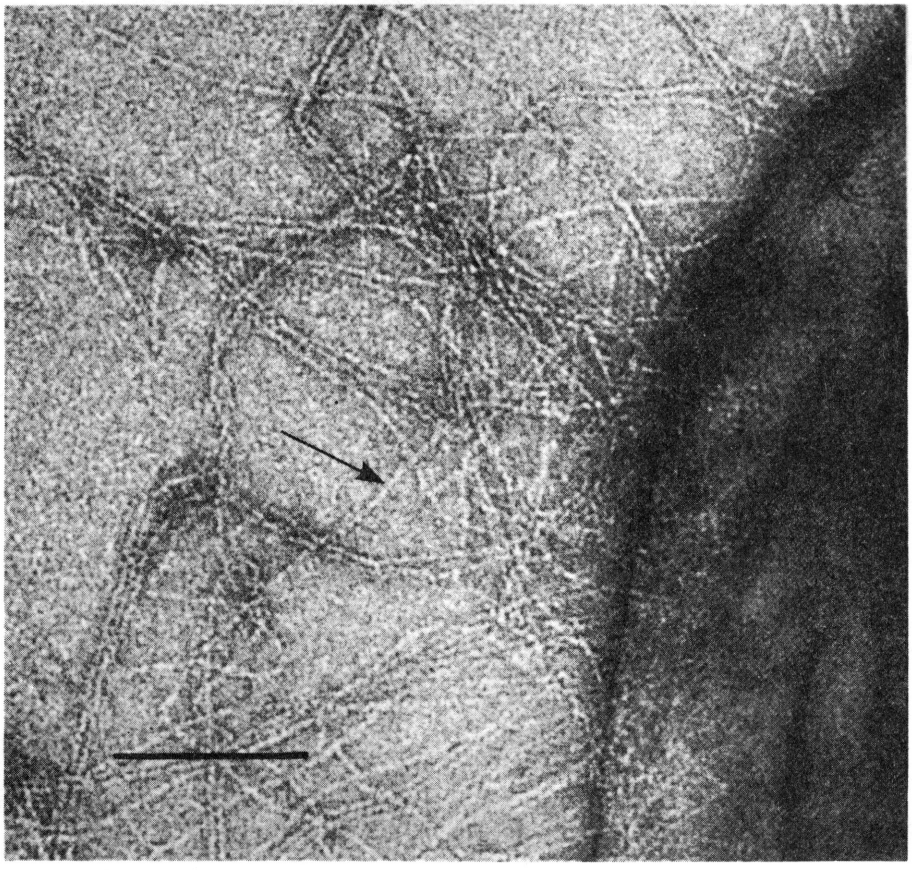

Fig. 26D

Table VII Extent and Type of Fimbriation of RAG-1, MR-481, RV-1 and RV23-4 Grown on Complex Medium and Ethanol Medium.

Strain	Complex medium[a]		Ethanol-grown[b]	
	thin	thick	thin	thick
RAG-1	++[c]	+	++	-
MR-481	-	+	-	-
RV-1	+++	-	+++	-
RV23-3	+++	-	+++	-

[a] Cells were grown to stationary phase (18 h) on brain heart infusion broth.

[b] Cells were grown to stationary phase (48 h) on PUM buffer supplemented with 2% (v/v) ethanol.

[c] Cells were stained with uranyl acetate and photographed in the electron microscope as described in the legend to Figure (-) denotes < 5 fimbriae per cell; (+) denotes 5-50 per cell; (++) denotes 50-200 per cell; (+++) denotes > 200 per cell

Table VIII Phage Sensitivity of RAG-1, MR-481, RV-1 and RV23-4

Strain[a]	Phage			
	215 H	Ø 11	AP-3	Ø 1
RAG-1	+[b]	+	+	-
MR-481	+	+	+	-
RV-1	+	-	+	-
RV23-4	+	+	-	+

[a] Cells were inoculated onto nutrient agar plates by the soft overlay technique. Ten µl drops of phage suspensions were placed on the plates and following 16 h incubation plates were examined for zones of lysis.

[b] Plus sign indicates phage sensitivity; minus sign indicates resistance.

3.6.5 Agglutinability by specific antibodies

Whole serum and purified antibodies (IgG fraction) obtained from rabbits inoculated with intact RAG-1 cells (see Materials and Methods) have been shown to agglutinate wild type RAG-1 cells (4). I compared the agglutinability of RAG-1, non-adherent mutant MR-481 and partial adherence revertants RV-1 and RV23-4 under various conditions. When compared to RAG-1, non-adherent mutant MR-481 cells were found to agglutinate extremely slowly or not at all, even in the presence of high antibody concentrations. In contrast, both RV-1 and RV23-4 were agglutinated three to ten times more rapidly than RAG-1 cells. Results of one experiment, in which agglutination of these four strains was compared with that of lysine auxotroph RAG-92 and its non-agglutinating derivative AB15 (see section 3.7), are shown in Table IX.

3.7 Non-agglutinating RAG-92 mutant AB15: Adherence properties and Growth on Hexadecane

In a study carried out in this laboratory (4), Bayer et al. used specific antibodies in order to select cell-surface mutants which were no longer agglutinated by these antibodies. Application of this technique using strain RAG-92, a lysine auxotroph derivative of RAG-1 (87), yielded several cell-surface mutants which were no longer agglutinated in the presence of antibody. One such mutant, AB15 (Table IX), was recently observed to be lacking thin fimbriae, both following growth on ethanol (E.A. Bayer, unpublished observations) and on complex medium (not shown). The apparent similarity between nonadherent mutant MR-481 and non-agglutinating mutant AB15 with regard to their poor agglutinability and absence of thin fimbriae suggested that these mutants may be closely related, despite the different techniques employed in their isolation (4,98). Thus strain AB15 was examined for (i) adherence to hydrocarbon, and (ii) growth on hexadecane.

Table IX. Agglutination Kinetics of RAG-1 and Derivative Strains by Antibodies Raised Against RAG-92 Cells[a]

Strain[b]	% Agglutination[c] after			
	6 h	22 h	28 h	46 h
RAG-1	9	69	84	92
MR-481	3	7	8	10
RV-1	89	96	95	96
RV23-4	80	98	92	91
RAG-92	9	53	67	84
AB15	0	1	3	5

[a] Antibody was obtained from rabbits inoculated with intact RAG-92 cells (see Materials and Methods).

[b] Cells were grown to stationary phase on brain heart infusion broth, washed and suspended in PUM buffer to an initial absorbance at 400 nm of 0.9 - 1.1.

[c] Percent decrease in optical absorbance at 400 nm (see Materials and Methods).

3.7.1 Adherence of Strain AB15 to hexadecane.

Adherence of AB 15 to hexadecane was compared with that of its parent strain, RAG-92, following growth in various media. No significant differences in adherence were observed for cells grown in complex media or on hexadecane vapours (Table X). Similarly, AB15 colonies on nutrient agar plates were replicated onto polystyrene (Fig. 27). However, following growth on ethanol, AB15 resembled MR-481 in its inability to adhere to hexadecane, and to polystyrene. Adherence of mutants MR-481 and AB15 to hexadecane following growth on minimal ethanol medium is compared with adherence of the respective parent strains, RAG-1 and RAG-92 in Fig. 28.

3.7.2. Growth of AB15 on hexadecane

Mutant AB15 and parent RAG-92 both grew well on minimal plates supplemented with hexadecane vapours or on acetate medium (supplemented with 100 µg/ml lysine) (Fig. 29a). However, AB15 was unable to grow on hexadecane in liquid medium under conditions similar to those described in sections 3.2.2 and 3.6 (Figs. 29b,30). When hexadecane was the sole carbon and energy source, RAG-92 grew, following a considerable lag; AB15, however, was unable to grow for at least six days. Similar results (not shown) were obtained when the inocula were obtained from cells grown on ethanol or on solid medium supplemented with hexadecane vapours.

3.8 Release by Shearing of a Cell-surface Antigen from Wild-type and Revertant Cells

In section 3.4, the effect of two mechanical techniques (sonic oscillation and shearing) on the adherence of RAG-1 to hydrocarbon was presented. While both treatments resulted in a reduction in adherence, prolonged sonic

Table X. Adherence of RAG-92 and AB15 to Hexadecane

Strain	Percent Adherence[a] of cells grown on		
	brain heart infusion broth[b]	nutrient broth[c]	hexadecane vapours[d]
RAG-92	100	97	89
AB15	95	94	83

a
Adherence to 0.2 ml hexadecane was performed as described in Materials and Methods.

b
Cells were grown overnight to stationary phase, washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of 1.45-1.52.

c
Cells were grown to early stationary phase, washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of 1.45-1.52.

d
Cells were grown on minimal plates supplemented with 100 µg/ml lysine and hexadecane vapours. Following 3 days growth, cells were scraped from the plates, washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of 1.15-1.20.

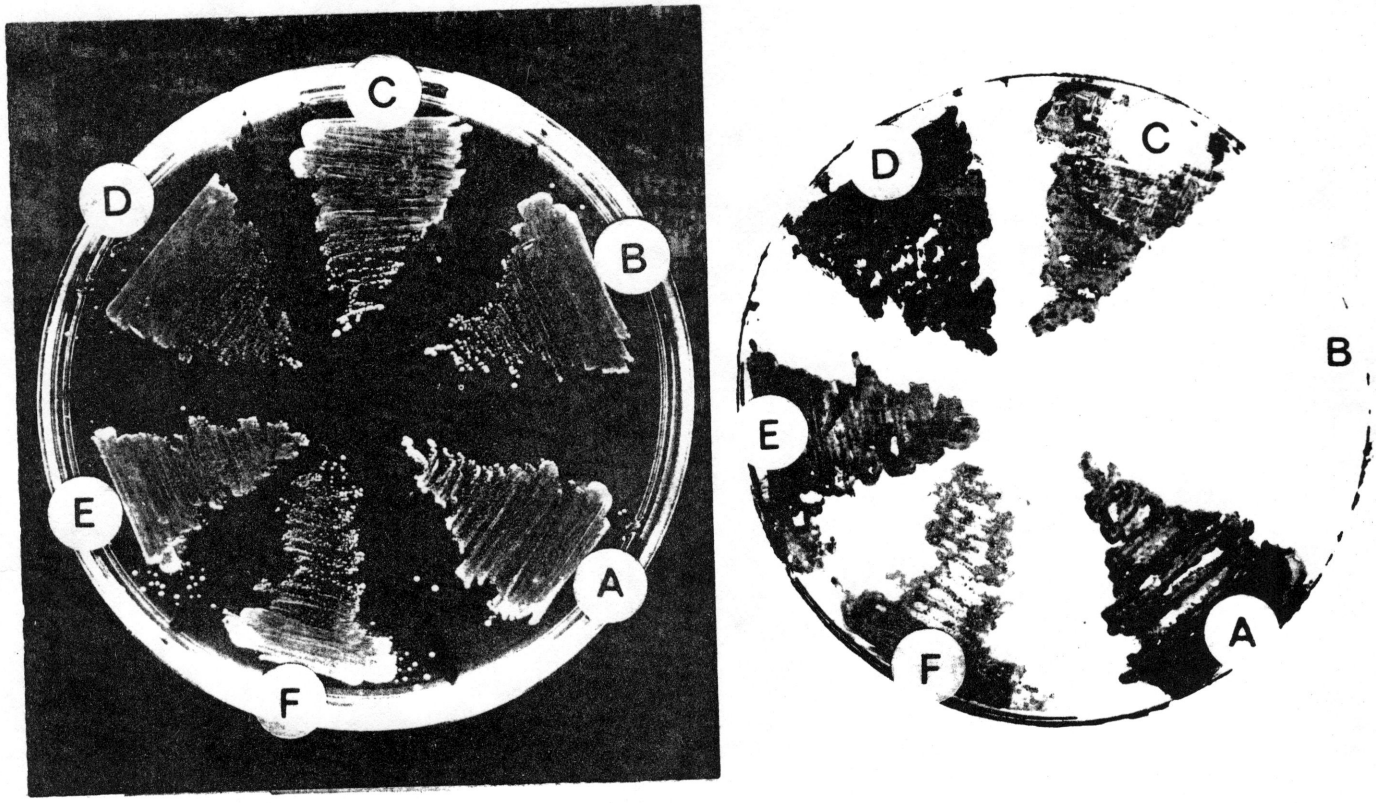

Fig. 27. Adherence of RAG-1 strains to polystyrene. Strains RAG-1 (A), MR-481 (B), RV-1 (C), RV23-4 (D), RAG-92 (E), and AB15 (F) were streaked onto a nutrient agar plate (left), and following 48 h incubation were replicated onto polystyrene (right) as described in the legend to Fig. 17. Only MR-481 cells were nonadherent under these conditions.

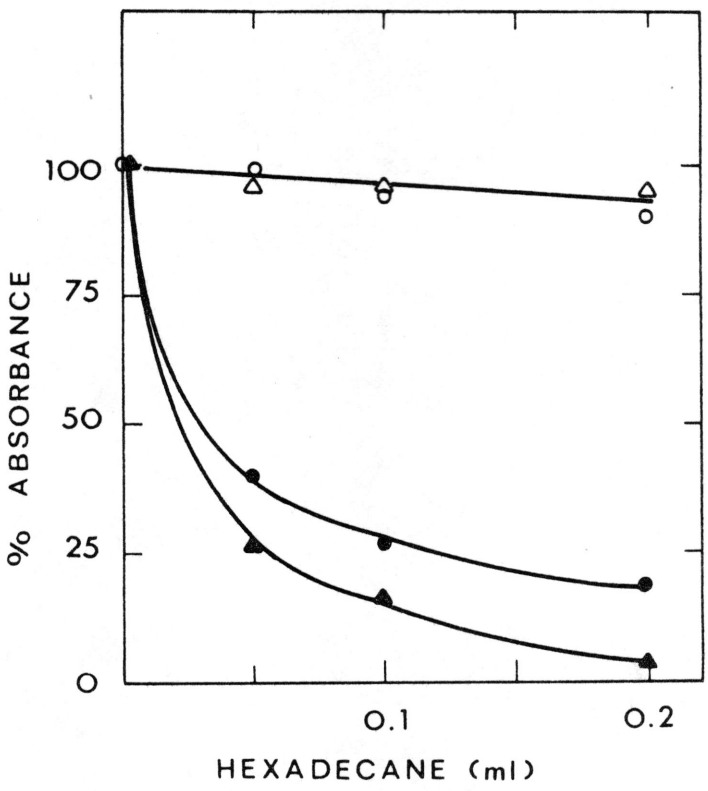

Fig. 28. Adherence of AB15 and MR-481 to hexadecane following growth on ethanol minimal medium. Strains AB15 (o), MR-481 (Δ), and the respective parent strains, RAG-92 (●) and RAG-1 (▲) were compared for adherence following 48 hr growth on 2% (v/v) ethanol minimal medium. Cells were harvested, washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of 1.35-1.55. Adherence to hexadecane was performed as described in Materials and Methods.

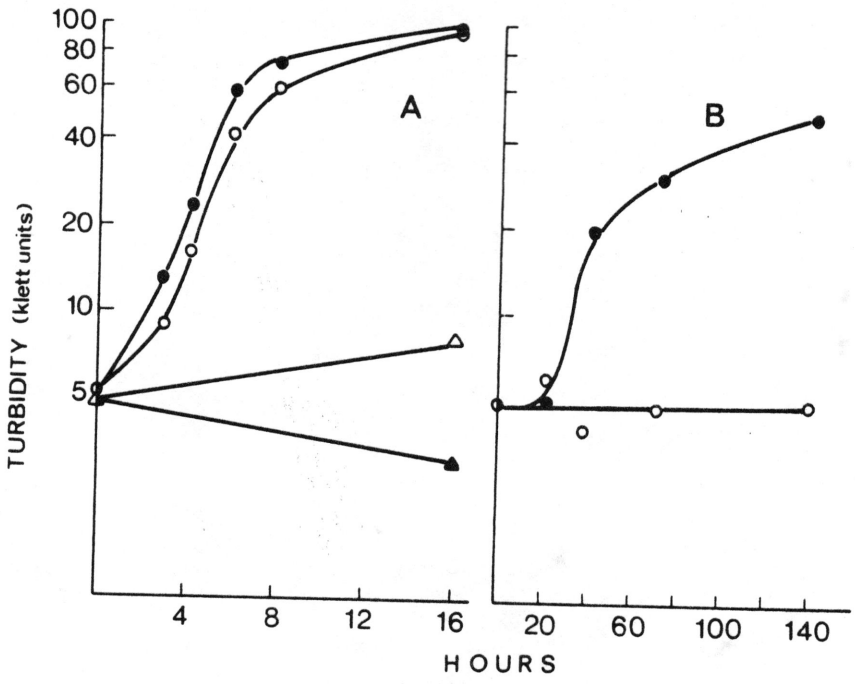

Fig. 29a. Growth of AB15 and RAG-92 in acetate medium. Stationary-phase cells were harvested from brain heart infusion broth, washed twice, and suspended in PUM buffer supplemented with 0.2% sodium acetate, and 50 µg/ml lysine. RAG-92 (●) and AB15 (o) cells were inoculated into 4.2 ml of acetate medium in Klett tubes to an initial turbidity of 5 Klett units. RAG-1 (▲) and AB15 (Δ) controls consisted of acetate medium, with no lysine added.

Fig. 29b. Growth of AB15 and RAG-92 in hexadecane medium. Klett tubes containing 4.2 ml of PUM buffer supplemented with 50 µg/ml lysine were inoculated with either RAG-92 (●) or AB15 (o) to an initial turbidity of 5 Klett units. The tubes were overlaid with 0.5 ml of hexadecane and incubated as described in the text.

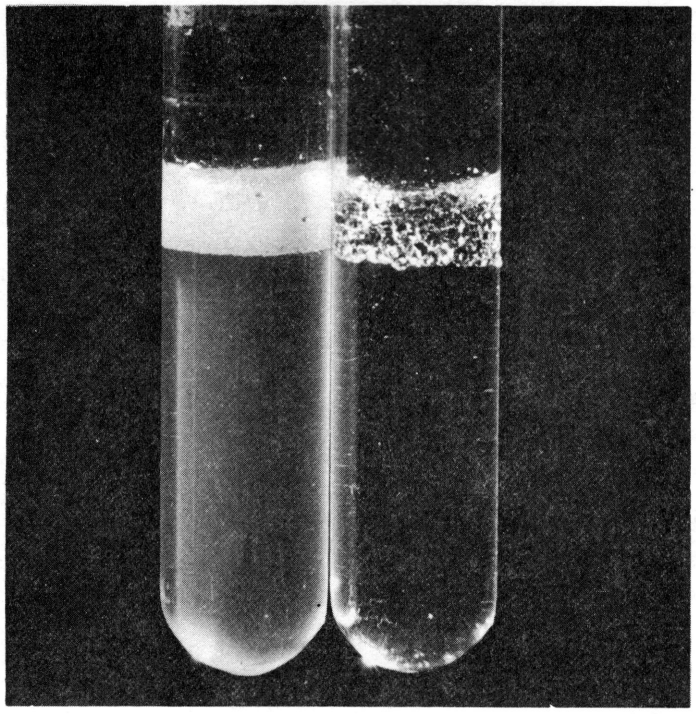

Fig. 30. Growth of RAG-92 (left) and AB15 (right) in hexadecane medium. The tubes were photographed after 4 days of incubation under the conditions described in the legend to Fig. 29 b.

oscillation of cells caused considerable cell lysis and released over 60 different proteins into the supernatant, as observed on SDS protein gels (not shown). We thus chose to examine the supernatants of the various strains following the more gentle shearing treatment applied to ethanol-grown cells. In order to visualize the bands, supernatants were first concentrated five-fold. In the crossed electrophoresis technique, the sample is run in the first direction in agarose not containing antibody, and is subsequently run at right angles into agarose gel which contains antibody. Initially, antiserum which had been preadsorbed with AB15 cells was employed, so that any antigens common to AB15 and wild type cells would not be precipitated, and the pattern easier to interpret (5). In the initial runs, a distinct solitary band was obtained with samples of RAG-1, RAG-92, RV-1 and RV23-4 samples; no bands were observable with samples of AB15 or MR-481. We then repeated the experiment, using nonadsorbed antibody raised against RAG-92 cells, expecting to see the appearance of antigens common to wild type and mutant cells. Similar results were obtained (Fig. 31), i.e., samples of wild type and partial revertant cells gave a common distinct band which was absent from MR-481 and AB15 samples, and no additional bands appeared. Results thus indicate that (i) both mutants MR-481 and AB15 lack a specific antigen which is released by the shearing treatment from the surfaces of wild type and partial revertant cells, and (ii) the sheared supernatant samples are relatively free of other antigenic cell surface components.

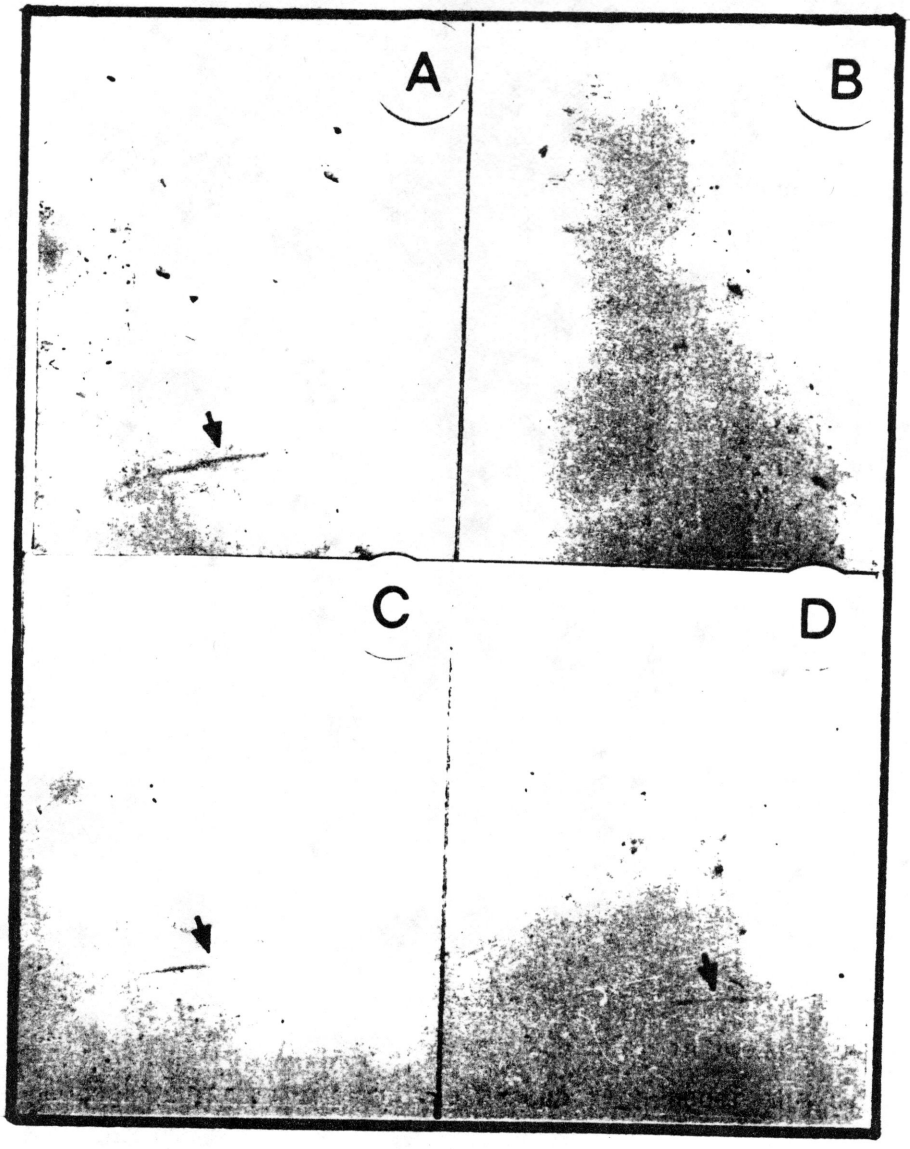

Fig. 31. Crossed immunoelectrophoresis of sheared supernatant fluids. Concentrated samples of supernatant fluid of cells sheared following growth on minimal ethanol medium were prepared as described in Materials and Methods. The crossed immunoelectrophoretic technique was performed as described in Materials and Methods. Samples were run in the first direction (left to right) in the absence of antibody, and then were run in the second direction (upwards) into gel containing non-adsorbed antibody raised against intact RAG-92 cells. Gels were subsequently washed, dried, pressed and stained. Samples: A - RAG-1; B - MR-481; C - RV-1; D - RV23-4. The presence of bands (peaks) is indicated by arrows.

DISCUSSION

4.1 Microbial adherence to hydrocarbons

Mudd and Mudd (73,74), in their classic papers written almost sixty years ago, observed that certain bacteria (e.g. Erythrobacillus prodigiosus, currently known as Serratia marcescens) have a tendency to partition at the oil:water interface, and attributed this ability to the hydrophilic:hydrophobic balance of the bacterial cell. More recently, Marshall and coworkers (66,67) have observed that tapered Flexibacter and Hyphomicrobium cells, as well as certain Rhizobium species adhere to liquid hydrocarbons in a stereospecific orientation, with one of the poles of the cell bound to the water:oil interface, and the remainder of the cell oriented perpendicularly into the bulk aqueous phase (69). Despite these published observations, microbial adherence to hydrocarbons has often been regarded, even in recent years, as a property unique to hydrocarbon-degrading bacteria and yeast (41,53,55).

The present study describes the development of a simple, quantitative technique for the measurement of microbial adherence to liquid hydrocarbon (94,96). The proposed method consists of mixing ("vortexing") a washed aqueous cell suspension in the presence of test hydrocarbons. Adherent cells partition at the aqueous:hydrocarbon interface and are quantitated by the corresponding decrease in turbidity of the bulk aqueous phase. In addition to the inherent simplicity of this technique, it differs from other quantitative methods for measuring adherence of bacteria and yeast to hydrocarbons (41,52,71,72) in two main details: (i) microbial adherence to hydrocarbon droplets is measured, rather than adherence of hydrocarbon droplets to cells; and (ii) surfactants, which may interfere with the

adherence process (74) are absent in this assay. Initial results using the method described here (94,96) demonstrated quantitatively that bacteria differ greatly in their affinity for the aqueous:hydrocarbon interface. Most laboratory strains tested adhered poorly or not at all to the test hydrocarbons. Among the strains which did adhere avidly were several hydrocarbon-degrading A. calcoaceticus strains, including RAG-1. However, several strains which do not metabolize paraffins, such as Serratia marcescens, Staphylococcus aureus, and Streptococcus pyogenes, also adhered to hydrocarbons. Thus, results clearly indicated that adherence to hydrocarbon is not a specific property of hydrocarbon-degrading microorganisms. Moreover, the low affinity of Pseudomonas aeruginosa towards the test hydrocarbons, despite its ability to grow on hydrocarbons, indicates that not all hydrocarbon-degrading bacteria adhere well to hydrocarbons (96).

While the results cannot be explained entirely within the context of petroleum degradation, they can be understood in terms of cell-surface hydrophobicity. Liquid hydrocarbons have a very low surface tension (2) and provide an extremely hydrophobic surface for attachment. A comparison of the data obtained in this study with other reports in the literature suggests that bacterial adherence to liquid hydrocarbon is a function of cell-surface hydrophobicity. Some examples are presented below:

(i) Pigmented Serratia strains have been implicated in a wide variety of hydrophobic interactions (13,20,44,57), such as their tendency to partition at the water:air interface. Kjelleberg et al. (57) found a high number of hydrophobic sites on the surface of Serratia cells as opposed to other bacterial species; moreover, these and other authors observed an increase in hydrophobicity of Serratia with increasing culture age and degree of pigmentation (13,44). Similar relationships were found in this study (Fig.6), using S. marcescens cells. (ii) Increased hydrophobicity of rough enterobacterial strains as compared to their smooth parents has been reported (23,54,65,86,106). Rough strains of both E. coli (94) and P.

mirabilis (98) exhibited increased affinity for the test hydrocarbons when compared to the corresponding smooth parental strains.

(iii) Adherence of Streptococcus pyogenes M-5, a pathogen and colonizer of the oral mucosa (1,95), to buccal epithelial cells has been postulated to occur via hydrophobic interactions (7,80,103). Hydrophobicity of other streptococcal strains has also been demonstrated (45,112). In this study it was found that S. pyogenes M-5 adheres well to the test hydrocarbons (Fig. 16). Moreover, in a recent study of adherent oral bacteria, various streptococci were found to adhere with high affinity to hexadecane (120).

(iv) Hydrophobic surface characteristics of Staphylococcus aureus strains were reported by several authors (32; T. Wadström, personal communication). In the present study, a local isolate of S. aureus adhered avidly to the test hydrocarbons, whereas a corresponding strain of S. albus was nonadherent (94).

(v) RAG-1 cells, grown under conditions for which adherence to hydrocarbons was optimal (i.e., cells grown in complex medium to stationary phase) exhibited other hydrophobic properties, such as copious frothing upon mixing of cell suspensions (indicative of adherence of cells to air bubbles), and adherence to solid hydrophobic surfaces, such as polystyrene (93). These properties were not observed with the nonadherent RAG-1 mutant, MR-481.

(vi) If microbial adherence to hydrocarbons is essentially due to hydrophobic interactions between cells and droplets, then surfactants should interfere with this process. Mudd and Mudd (74) observed that adherence of cells to oil was hindered when detergents were present. Similarly, we found that adherence of RAG-1 to hexadecane was inhibited and reversed in the presence of surface active agents, such isopropanol (Fig. 1) and emulsan (Table V).

These results, taken together, demonstrate that bacterial adherence to hydrocarbons, as described in this report, can be employed to study the hydrophobic characteristics of various microorganisms. While a detailed analysis of other techniques which have been or are being used to determine bacterial hydrophobicity is beyond the scope of this work, a brief description of several quantitative approaches is presented in the following paragraphs for comparative purposes.

A common method for measuring cell-surface hydrophobicity of bacteria is to measure their retention on columns of hydrophobic gels (hydrophobic interaction chromatography) usually octyl- or phenyl- Sepharose (25,26,79,118). Wadström and coworkers (35a,104,115) have demonstrated increased hydrophobicity of pathogenic E. coli strains on this basis. Unfortunately, most of these studies fail to compare the retention of bacteria by these gels with their retention time by the untreated Sepharose matrix. Thus, some reported cases of bacterial retention on hydrophobic gels may be due to other factors, such as cell size and physical entrapment (27).

van Oss and coworkers have studied the hydrophobic characteristics of cells by adapting the contact angle technique to layers of bacteria. In this method, a drop of saline is applied to a layer of bacteria, and the angle of contact determined. These authors were able to demonstrate that bacteria which were more hydrophobic than phagocytes were ingested, whereas more hydrophilic cells evaded phagocytosis (23,113). While this method has been subject to some criticism (76), it enables an estimation of the overall surface tension of the bacterial cell surface (77).

Another means of measuring bacterial hydrophobicity is by partitioning of cells in two-phase systems. Reed and Rice (89) established quantitatively that extremely hydrophobic bacteria, such as Mycobacteria, tend to pass into the oil phase when mixed in an oil:water mixture. These authors did

not observe partioning of cells at the oil:water interface (as has been done in this study), probably because of their limited choice of test organisms. A similar method involves the partitioning of bacteria in a two-phase aqueous system (38,65,86,106), dextran and polyethylene glycol (PEG). The PEG rich phase can be made increasingly hydrophobic by covalent binding of alkyl residues. This system enables measurement of much smaller differences than those observed by Reed and Rice (89), such as the difference in hydrophobicity of rough and smooth strains (65,86).

Yet another approach to the study of bacterial hydrophobicity is measurement of binding of amphipathic molecules to the cell surface. These hydrophobic molecular probes are usually anionic, to avoid attractive forces between the negatively-charged bacterial surface and the probe (32,45,56). Recently, Kjelleberg and coworkers have studied the binding of ^{14}C-labelled dodecanoic acid to bacterial cells (56). While this technique provides important data on the number of hydrophobic sites on the cell surface and their relative affinities, it may not provide a clear picture of the ability of the cell as an entity to adhere via hydrophobic interactions. Molecular probes may penetrate the outer surface of the cells and thus bind to sites which are buried and cannot participate in adherence phenomena. Furthermore, a cell whose hydrophobic sites are tightly clustered, may adhere with higher affinity to hydrophobic surfaces, than a cell with an equivalent number of molecular binding sites which are evenly distributed over the cell surface. Bacteria which adhere at the oil:water interface via a polar region (66-69) may be an example of a largely hydrophilic cell, with a concentration of exposed hydrophobic components at one site.

From the above, it is clear that there is no dearth of methods for measuring bacterial hydrophobicity. Since the methods differ in their approach to the measurement of bacterial hydrophobicity and in the

quantitative parameters which they provide, a combination of several methods, rather than a single technique, may provide the most reliable and revealing picture of cell surface hydrophobicity. Bacterial adherence to liquid hydrocarbons has a number of advantages over other methods: (i) it is simple and inexpensive; (ii) quantitative results are rapidly obtained; and (iii) elaborate equipment is not required. In addition, adherence to liquid hydrocarbons provides a relatively defined model system for studying adherence of bacteria to complex biological surfaces known to exhibit hydrophobic characteristics, such as epithelial cells (95) and teeth (120).

In addition to these applications, bacterial adherence to hydrocarbons can be employed to separate heterogeneous cell populations, i.e., hydrophobic spores from nonadherent vegetative cells (Fig. 32;12,16). Moreover, in strains which adhere avidly to hydrocarbon, nonadherent mutants may be selected by sequential mixing and growth procedures. This approach enabled the isolation of nonadherent RAG-1 mutant MR-481 (Section 3.2.1). In principle, mutants which are more adherent than the wild type cells may also be selected by enrichment of cells which partition with the highest affinity at the aqueous:hydrocarbon interface.

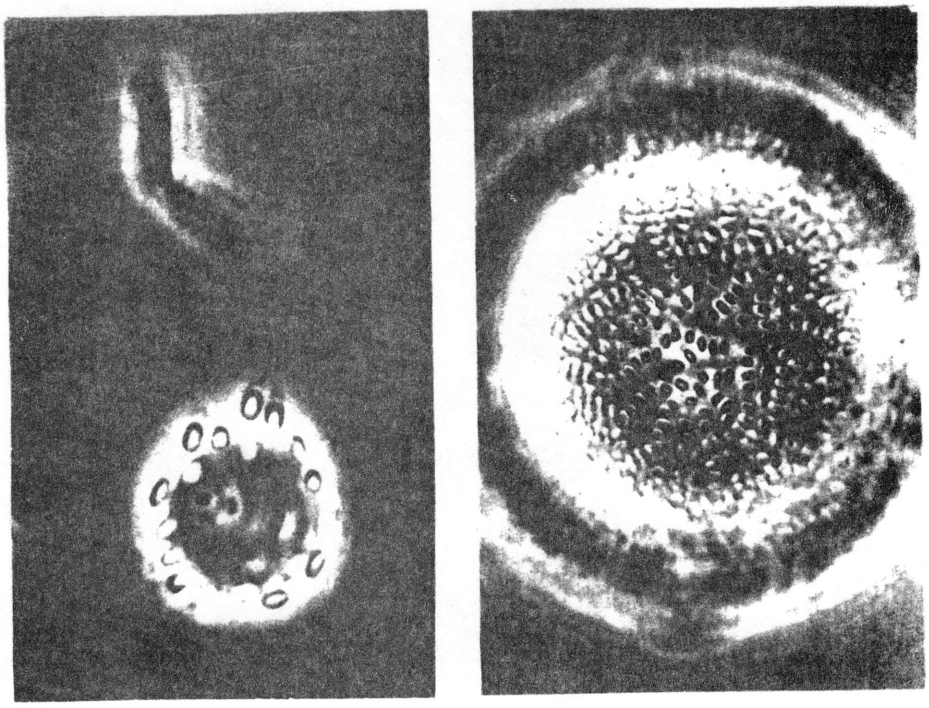

Fig. 32. Adherence of Bacillus cereus endospores to hexadecane. When a mixture of vegetative and endospore cells of B. cereus, scraped from a nutrient agar plate following 72 h incubation at 30°C and suspended in PUM buffer, was mixed in the presence of hexadecane, as described in the legend to Fig. 1, spore cells adhered to the hydrocarbon droplets while vegetative cells remained in the bulk aqueous phase. Right: endospores adhering to a large hexadecane droplet (x 1100). Left: nonadherent vegetative cell adjacent to small hexadecane droplet covered with endospores (x 3200).

4.2 Role of adherence in growth of RAG-1 on hexadecane.

The role of adherence in growth of microorganisms on oil has been a subject of controversy during the past decade (41). Although adherence of cells to oil droplets has frequently been observed during hydrocarbon fermentations (41,68,70,75,76), there has been no direct evidence that adherence is an essential aspect of microbial growth on hydrocarbons. Studies presented here (97) have shown that adherence to liquid hydrocarbons is a general phenomenon, mediated by hydrophobic interactions, and shared by bacteria which do not degrade hydrocarbons. Moreover, P. aeruginosa cells adhered poorly to the test hydrocarbons, even following growth on hexadecane (Fig.8;96). Certain investigators have argued that microbial growth on hydrocarbons is due to emulsification of the water-insoluble substrate rather than by direct contact between cells and hydrocarbon droplets (19). A. calcoaceticus RAG-1 is an interesting microorganism in this regard because it both adheres avidly to hydrocarbons and produces an extracellular emulsifying agent, emulsan, which has been extensively characterized. Emulsan is an anionic heteropolysaccharide (125) containing fatty acid side chains (8), with an average molecular weight of 9.9 x 10^5 and an axial ratio of about 60.

Isolation of a mutant strain of A. calcoaceticus RAG-1 deficient in its ability to adhere to hydrocarbon enabled an examination of the role of adherence in the growth of RAG-1 on hexadecane. The nonadherent mutant strain, MR-481, failed to grow on hexadecane under the moderate agitation conditions which supported growth of wild type cells. Two obvious explanations for the inability of MR-481 to grow on hexadecane, insufficient oxygen and a faulty hydrocarbon-metabolizing enzyme system, were excluded by the following data: (i) MR-481 and RAG-1 cells grew with similar kinetics on acetate medium under conditions identical to those employed for growth on hexadecane; (ii) MR-481 was able to grow on hexadecane medium following addition of an emulsifier; and (iii) MR-481 was

able to grow on solid medium supplemented with hexadecane vapours as sole carbon and energy source. These data demonstrate that adherence is a prerequisite for growth of RAG-1 on hexadecane in the absence of strong mechanical agitation or emulsification of substrate.

Both RAG-1 and MR-481 produce emulsan, which, when added exogenously, enabled growth of non-adhering MR-481 cells on hexadecane. The observation that MR-481 does not grow on hexadecane even though it produces normal amounts of emulsan can readily be explained by considering emulsification as a cell-density dependent phenomenon (59,91). At the low inoculum employed (5×10^7 cells/ml), the concentration of emulsan which the cells produce is too low to emulsify the hydrocarbon and permit growth.

These studies suggest that adherence is a prerequisite for microbial growth on liquid hydrocarbons under two conditions: low cell density and limited emulsification. The ability of MR-481 to grow on hexadecane following emulsan addition demonstrates that the presence of extracellular emulsifying agents can enable growth of nonadherent bacteria on hydrocarbons. The poor adherence of P. aeruginosa PAS279 to hydrocarbon (Fig.8) suggests that its growth, similar to that of MR-481, depends on dispersion of the hydrocarbon phase by extracellular emulsification or vigorous mechanical agitation. In most natural environments, conditions of low cell density and limited emulsification prevail; thus, the growth of poorly-adhering Pseudomonas strains in shake flasks and fermentors carried out in the presence of extraneous emulsifiers or vigorous agitation (19,46,114,117) probably does not reflect growth in the open aqueous environment, where emulsifier and dispersed hydrocarbon would tend to diffuse away from the cell.

Adherent microorganisms, on the other hand, would remain in intimate contact with their substrate and would thus be availed of an adequate supply of hydrocarbon whether in a closed or open system. This basic difference between these two modes of growth on hydrocarbon is illustrated in Fig. 33.

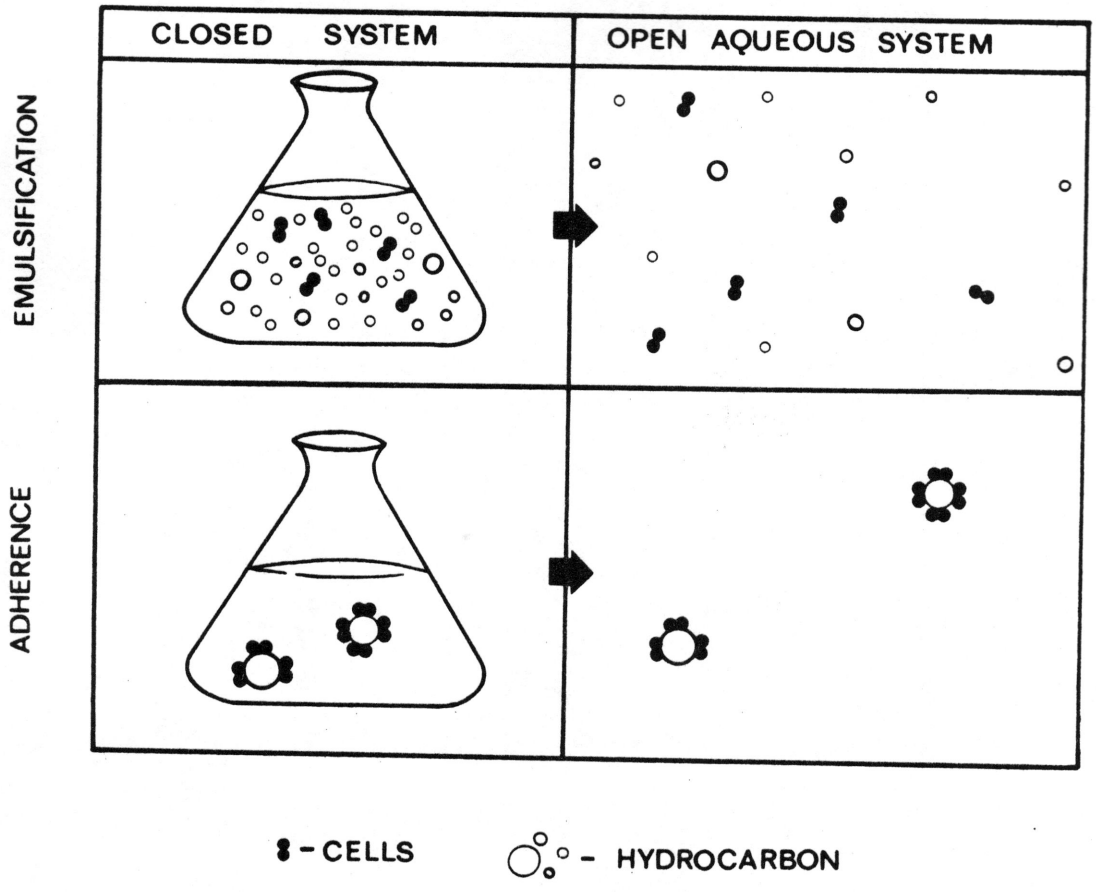

Fig. 33. Model: Adherence versus emulsification in microbial growth on liquid hydrocarbons. While the availability of emulsified hydrocarbon in a closed system (e.g., fermentor or shake flask) may enable growth of nonadherent cells (top left), the introduction of such a mixture into an open aqueous environment would result in diffusion of the dispersed hydrocarbon away from the cells, thereby lowering substrate availability and restricting growth. Adherent cells, transferred from a closed (bottom left) to an open aqueous environment (bottom right), would remain in direct contact with the growth substrate, enabling growth.

An interesting phenomenon which has also been observed by other investigators during growth of A. calcoaceticus on hexadecane is the appearance of free cells in the aqueous phase. Neufeld et al. (76) reported that the appearance of free cells coincides with an abrupt decrease in the hydrophobicity of the cells. In the present study, no evidence was found that the cells which appeared in the aqueous phase during growth had lost their ability to adhere to hydrocarbon. RAG-1 cells, harvested from the aqueous phase and washed following 54 h of growth still adhered avidly to hexadecane (Table II). The appearance of unbound RAG-1 cells in this report can be best explained by emulsan production during growth on hexadecane (92). The presence of emulsifiers in hydrocarbon fermentations is accompanied by a decrease in interfacial tension between the hydrocarbon and aqueous phases (70,76) which may, in turn affect the thermodynamic favorability of cell partitioning at the interface. In support of this hypothesis, addition of emulsan to the adherence assay resulted in a release of the majority of RAG-1 cells bound to the hexadecane (Table V).

What, then is the role of emulsan in adherence and growth of RAG-1 on hydrocarbon? One of the initial working hypotheses of this study was that emulsan, bound to the cell surface prior to its release functioned as a hydrophobic ligand and enabled the cells to adhere to the hydrocarbon surface. A number of observations during the course of this investigation indicated that this hypothesis is not valid: (i) nonadherent mutant MR-481 produced normal amounts of emulsan; (ii) RAG-1 mutants, deficient in emulsan synthesis (87), adhered to hydrocarbons with affinities similar to those of the wild type cells (O. Pines and M. Rosenberg, unpublished data); (iii) conditions which brought about the release of cell-bound emulsan (e.g., chloramphenicol treatment (99)) did not reduce the ability of RAG-1 cells to adhere to hexadecane.

The role of extracellular emulsan in growth of RAG-1 on hydrocarbons remains unclear at present. Three possible functions are currently under

consideration: (i) Emulsan permits growth on crude oil by enabling cells to cope with toxic compounds present in petroleum (116); (ii) Emulsan plays a role in releasing bound cells from the aqueous:hydrocarbon interface (97), thus enabling cells to escape from an overcrowded interface to new habitats (E. Rosenberg, personal communication); and (iii) Emulsan, bound to the cell surface, participates in hydrocarbon assimilation and uptake. The possibility also exists that emulsan is a capsular component of RAG-1 which is released only under laboratory growth conditions and has no natural role as a cell-free polymer. A capsular component released from A. calcoaceticus BD 413 during growth has been shown to possess emulsifying activity, similar to that of emulsan (50).

The importance of adherence in growth of RAG-1 on hexadecane was further emphasized in experiments dealing with the isolation and properties of the partial revertant strains. The prolonged incubation of nonadherent mutant MR-481 in hexadecane medium selected for partial revertant clones which exhibited renewed ability to adhere to polystyrene and partial ability to adhere to hydrocarbons. One such clone, RV-1, upon further growth in hexadecane medium, gave rise to a second partial revertant, RV23-4, which exhibited increased affinity for the test hydrocarbons, in addition to its ability to adhere to polystyrene. The ability of these two strains to grow on hexadecane under the conditions studied correlated well with their adherence ability. Thus, RV-1, which adhered rather poorly to hexadecane, grew to about one-third of the yield of RAG-1, whereas RV23-4, which exhibited a higher affinity for hexadecane, but still considerably less than that observed for RAG-1, grew to approximately half the cell density of RAG-1.

Results recently obtained with mutant AB15, a non-agglutinating mutant of RAG-92 partially deficient in adherence properties, suggest that adherence, however important, may not in itself be sufficient to allow growth on hexadecane in the absence of thin fimbriae. This possibility is discussed in Section 4.5.

4.3 RAG-1 Adherence to Hydrophobic Surfaces: Specific versus General Interactions

Some examples of bacterial adherence in the literature are ambiguously termed either "specific" or "general" (6,9). One example is the adherence of MS^+ (mannose-sensitive) strains to surfaces bearing mannose residues (81). Mannose-sensitive adherence has been reported to involve adhesins on the bacterial surface which specifically recognize mannose residues and can be blocked by specific analogs (e.g. α-methyl mannoside) (35,81,85). However, whereas a major biological role of such adhesins probably involves adherence to mucosal cells (33,81,85), MS^+ strains adhere in a non-specific manner to various other surfaces, such as yeast cells and red blood cells, via the same mechanism. Conversely, hydrophobic interactions involved in bacterial adherence phenomena have often been termed "non-specific" or "general" (9,69), presumably because (i) they are inhibited by a wide range of surfactants, rather than by specific molecules, and (ii) complementary molecular configurations are apparently not required. Nevertheless, bacterial hydrophobicity and adherence to hydrocarbons are,as has been shown in this and other studies, species- and strain-specific properties. Bacterial hydrophobicity is mediated by specific cell-surface components which are probably absent in most laboratory strains, and which are expressed only under certain growth conditions. Moreover, bacterial hydrophobicity plays a role in very specific biological adherence phenomena, including adherence to mucosal cells (95), phagocytosis (113), and growth of microorganisms on insoluble, hydrophobic substrates, such as hydrocarbons (97). These examples emphasize the importance of further defining the terms "specific" or "general" when discussing bacterial adherence phenomena. Specificity (or lack thereof) can exist on at least four levels: (i) Specificity of adherence mechanism - Does adherence occur via complementary stereospecific configurations and recognition on a molecular level?; (ii) Strain- or species- specificity - Is the adherence phenomenon under discussion restricted to particular strains or species?;

(iii) Cell-surface specificity - Is the adherence phenomenon mediated by a specific component (or specific components) on the bacterial cell surface?; and (iv) Substratum specificity - Is adherence limited to the particular attachment surface (or interface) under consideration?

The adherence phenomena of RAG-1, as observed in this study, are best understood in the above context. Thus, whereas the adherence phenomena of RAG-1 studied here probably do not involve specific stereochemical complementarity (as evidenced by lack of substratum specificity), adherence of RAG-1 cells is mediated by specific surface components. Moreover, the heterogeneity of adherence properties manifested by different Acinetobacter strains suggests that these adherence components are specific for certain Acinetobacter strains and not for others.

4.4 Approaches to the Study of Hydrophobic Adherence Phenomena

The study of hydrophobic adherence phenomena is hampered by a basic difficulty: that of non-specific inhibition. Thus, while much can be learned about lectin-like interactions by introduction of mono- or oligosaccharides which may or may not interfere with the adherence process (81), relatively little can be gleaned by a similar approach to adherence mediated by hydrophobic interactions. For example, the inhibition of adherence of RAG-1 to hexadecane and the removal of bound cells by emulsan (Section 3.4.3) does not imply that cell-bound emulsan contributes to the adherence process; other surfactants, such as isopropanol (Fig. 1), have a similar effect.

Two general approaches are currently employed for the study of hydrophobic interactions in bacterial adherence. In the first approach, the physical properties of the cell surface as a whole (such as contact angle studies) are used to predict bacterial adherence phenomena (113). The drawback of this approach is that it treats the cell surface as being homogeneous and

may thus largely ignore the possible contribution of specific, or localized structures, such as holdfasts, or fimbriae (33,66-69,85), to adherent properties in general and hydrophobic interactions in particular.

The second approach is to attempt to identify and subsequently characterize specific cell-surface components involved in mediating hydrophobic interactions. Much is known, for example, about K88, K99 and CFA antigens on E. coli cells which promote adherence and colonization of mucosal cells; these filamentous antigens have been reported to mediate hydrophobic interactions of these strains as measured by hydrophobic interaction chromatography (35a,85,104,115). In the present study, a combination of genetic and immunological approaches has enabled the identification of one of the cell surface components of RAG-1 contributing to its adherent properties. This topic is discussed in the following section.

4.5 The Role of Thin Fimbriae in Mediating RAG-1 Adherence to Hydrocarbon and Growth on Hexadecane

Bacterial fimbriae (pili) play a role in many biological phenomena, including pathogencitiy (33,35a,81,85,102,104,115), various adherence phenomena (33,81,85,121), pellicle formation (33), and twitching motility (42). Fimbriae of enterobacterial species have been classified into groupings by various researchers (33,85); thus, for example, type 1 fimbriae refer to filaments of approximately 7.0 nm in diameter which exhibit mannose-binding properties (35).

Various fimbriae have been purified, and are found to consist of protein subunits (35,85). Such subunits contain a high percentage of hydrophobic amino acids. Hydrophobic interactions of pathogenic E. coli strains have been attributed to the presence of specific fimbrial antigens (35a,104,115).

RAG-1 cells possess at least two distinct fimbriae: (i) a thick variety, about 6.5 nm in diameter, which may be involved in twitching motility (42); and (ii) a thin variety, about 3.5 nm in diameter, similar to thin fimbriae observed in other strains of Acinetobacter (42).

Mutant MR-481, a nonadherent mutant of RAG-1 and AB15, a nonagglutinating mutant of lysine auxotroph RAG-92 were both observed to be devoid (or almost completely devoid) of thin fimbriae, as observed in numerous electron micrographs. These two mutants, though isolated by completely different procedures (4;Section 3.2.1), were similar in several additional properties: (i) both mutants were very poorly agglutinated by antibody raised against intact RAG-92 cells (Table IX); (ii) both mutants were nonadherent following growth on ethanol (Fig.28); (iii) Both mutants were unable to grow on hexadecane in liquid medium under the conditions described in Sections 3.2.2 and 3.7.2, despite the ability of both strains to grow on hexadecane vapours; and (iv) both mutants lacked a specific cell-surface antigenic component which could be released by shearing from wild type cells (Section 3.8). Mutants AB15 and MR-481 differed in two important aspects: (i) AB15 adhered to hexadecane, similar to the parent strain, following growth on complex medium and on hexadecane vapours (Section 3.7.1), whereas mutant MR-481 was nonadherent (Fig. 11); and (ii) AB15 cells grown on nutrient agar plates adhered to polystyrene whereas similarly grown MR-481 cells were nonadherent (Fig.27). This conditional appearance of adherent properties in mutant AB15 was not the result of conditional expression of thin fimbriae.

Prolonged incubation of MR-481 cells on hexadecane in liquid medium led to the sequential isolation of two partial adherence revertants, RV-1 and RV23-4. Both strains differed from RAG-1, MR-481, and each other, as evidenced by their phage sensitivities (Table VIII), and comparison of their adherent properties (Fig.23). Both strains (RV-1 and RV23-4) exhibited (i) a copious number of thin fimbriae as observed by electron microscopy (Section 3.6.3); and (ii) extremely rapid antibody-mediated agglutination, compared with that of RAG-1 cells (Table IX). Both partial

revertants yielded, upon shearing treatment, the same antigenic band which was present in wild type RAG-1 and RAG-92 cells, but absent from mutants MR-481 and AB15 (Fig.31).

These results, taken together with the loss of adherence to hexadecane following shearing of ethanol medium-grown RAG-1, RAG-92 and 16-tlu cells (Section 3.4.1) suggest the following conclusions:

(1) The thin fimbriae are the major antigen responsible for the agglutination of RAG-1 cells.

(2) The thin fimbriae are responsible for the adherence to hexadecane of RAG-1 following growth on ethanol minimal medium (this explains the non-adherence of ethanol-grown AB15 cells), and can enable adherence of cells to polystyrene (hence, the adherence of RV-1 to polystyrene).

(3) Appearance of partial adherence characteristics in strain RV-1 (adherence to polystyrene; slight adherence to the test hydrocarbons) is at least partly due to the appearance of numerous thin fimbriae which may not be identical to the thin fimbriae of RAG-1 cells, but do resemble them as observed by electron microscopy, in antigenicity, and in electrophoretic mobility, as shown by crossed immuno-electrophoresis.

(4) Other adherence factor(s) are present on the surface of wild type RAG-1 cells grown on complex medium or hexadecane, hence (i) the greatly increased adherence to hydrocarbon of cells grown on complex medium or hexadecane with respect to ethanol-grown cells (Fig.5); (ii) the conditional adherence characteristics of AB15; and (iii) the inability of shearing to significantly reduce adherence to hexadecane of RAG-1 cells grown on complex medium (Section 3.4.2).

These additional components greatly increase the affinity of RAG-1 cells for hydrocarbon and can mediate adherence to polystyrene, even in the absence of thin fimbriae (thus AB15 cells are able to adhere to polystyrene following growth on nutrient agar, as shown in Fig.27). These additional adherence component(s) are absent from the surface of MR-481 cells; however, one of them may be present in strain RV23-4. Alternatively, the increased adherence of RV23-4 with respect to RV-1 may be due to the exposure of a hydrophobic component which is buried on the surface of RAG-1. Other cell surface components which may be involved in the adherence properties of RAG-1, as studied here, include lipid molecules, such as waxes (17,110), and amphipathic cell surface polysaccharides (122).

(5) Adherence to hexadecane may be a necessary but insufficient factor in enabling growth of RAG-1 on hexadecane under the conditions described in Section 3.2.2. The inability of AB15 to grow on hexadecane, even when the inoculated cells were initially adherent (Section 3.7.2) suggest that the presence of thin fimbriae may be crucial for growth on hexadecane in liquid culture, even when the cells are adherent. This possibility requires further examination, since the parent RAG-92 cells exhibited poor growth on hexadecane, as compared to RAG-1 cells (Section 3.2.2). This anomoly may be related to the lysine auxotrophy of RAG-92.

(6) RAG-1 cells harvested from complex medium in early exponential phase adhered very poorly to hexadecane and epithelial cells (Table III; Fig.5). Electron micrographs of these cells (not shown) reveal that they have very few thin fimbriae. The additional adherence factor(s) are probably also absent from the surface of RAG-1 cells under these conditions.

The basic differences between the two parental strains, RAG-1 and RAG-92, and cell-surface mutants MR-481 and AB15, following growth on complex medium and minimal ethanol medium are summarized in Fig. 34.

In the present investigation, only one of several factors which may determine adherence of RAG-1 to hydrocarbons and other hydrophobic surfaces has been identified. However, the correlations obtained between the presence of thin fimbriae and growth on hexadecane in liquid culture suggest that the thin fimbriae may be the most important adherence factor in enabling growth on hexadecane. The appearance of a single band in the crossed immunoelectrophoresis of the supernatant fluid of RAG-1 cells subjected to shearing treatment following growth on ethanol minimal medium, even when run against antibody which had not been preadsorbed with AB15 cells, suggests that this supernatant fluid is relatively free from other surface antigens, such as emulsan, and that a scale-up of the shearing process can be used to obtain a pure preparation of thin fimbriae for further study and characterization.

In a separate study (5), crossed immunoelectrophoresis of spent ethanol medium following growth of RAG-92 cells revealed the presence of several peaks, including one corresponding to emulsan, and a band which corresponds to the one observed in this study (Fig. 31). This latter band was present in spent medium of emulsan-deficient strain 16-tlu, but absent from the spent growth medium of AB15. These results are in complete agreement with the data presented here.

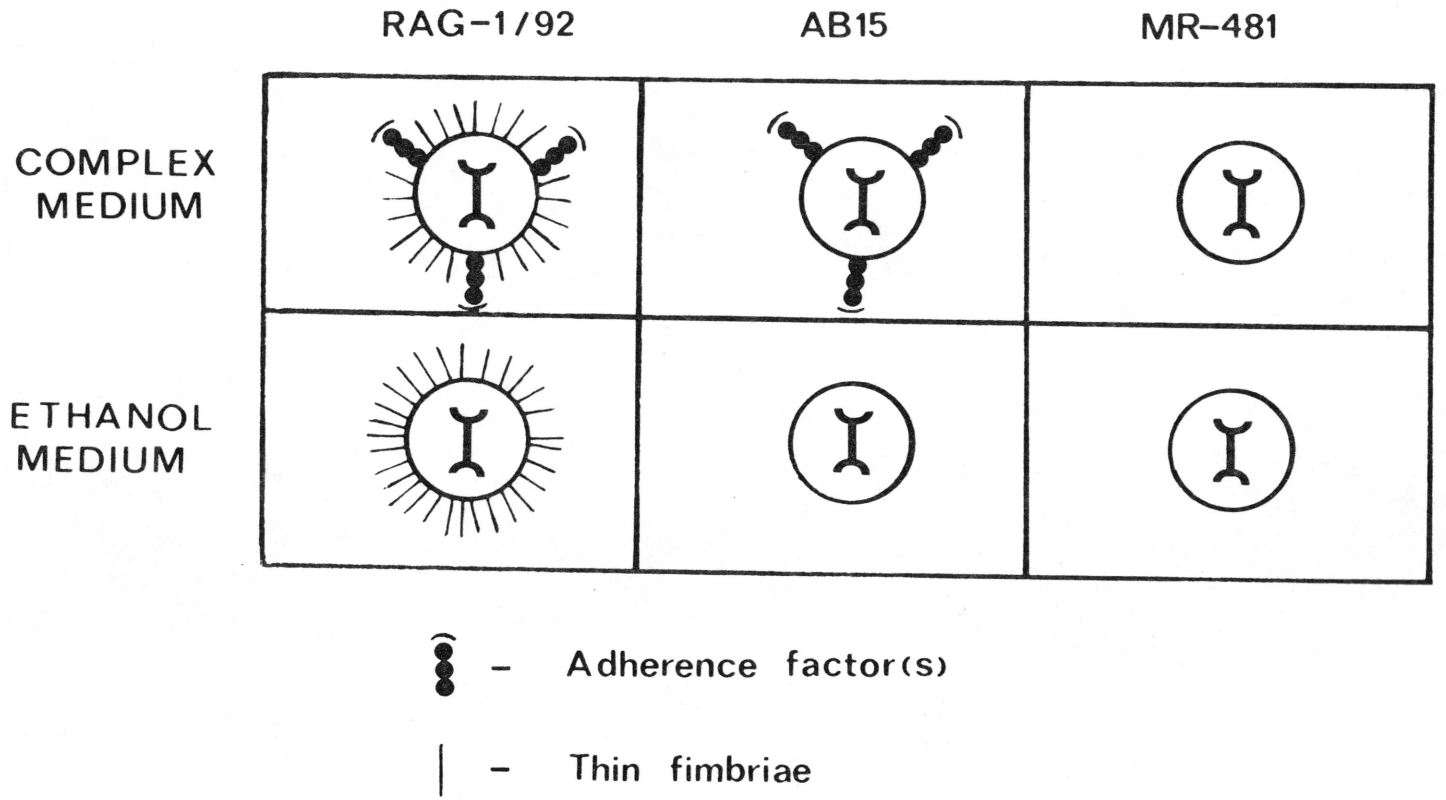

Fig. 34. Model: Adherence components of RAG-1, RAG-92, and mutants MR-481 and AB15 following growth on complex medium and minimal ethanol medium. Both mutants MR-481 and AB-15 lack the thin fimbriae present on wild type cells. The additional adherence factor(s) which are present on wild type cells following growth on complex medium are present in complex-medium grown AB-15 but absent from the surface of MR-481.

4.6 Adherence to hydrophobic surfaces: Liquid-liquid vs. Liquid-solid interfaces

In a previous study in this laboratory, I. Kirschner Zilber et al. (55) studied parameters affecting the adherence of marine pseudomonad UP-2 to a solid paraffin, n-tetracosane, during growth. In the present investigation, adherence and growth of RAG-1 on liquid hydrocarbon have been studied. Since these two studies employed different strains, growth media, and adherence assays, any comparison would be difficult. Moreover, while adherence to solid and liquid hydrocarbons may involve similar mechanisms, it appears that they are not identical processes. The release of cells from the hexadecane droplets upon solidification (Fig.22) suggests that following this phase transtition, adherence under the conditions studied is no longer favourable thermodynamically. One possibility, suggested by P. Rutter, is that adherence of RAG-1 cells to liquid hexadecane involves a gross deformation of the interface, similar to that proposed by Mudd and Mudd (74), whereas much less deformation of the interface would be possible in the case of adherence to a solid surface. An experimental approach to determine the validity of this hypothesis would be of great value, since knowledge of the degree of deformation of the aqueous:hydrocarbon interface by the cells could provide an estimate of the energy of adherence (111). Similarly, while most cases of adherence to polystyrene can be correlated with adherence to liquid hydrocarbons (93), several important exceptions were found. For example, RV-1 cells adhere well to polystyrene (Fig.27), but poorly to hexadecane (Fig. 23). Similarly, A. calcoaceticus BD4 cells grown on nutrient agar are replicated onto polystyrene, but do not adhere to hexadecane (N. Kaplan, unpublished observation).

4.7 Bacterial hydrophobicity: A general microbial principle

Investigations into biological roles of bacterial hydrophobicity and the prevalence of microorganisms which exhibit hydrophobic surface properties, both in the open environment, and within the host, are currently being conducted in various laboratories. Dahlbäck et al. (25) have shown that a high percentage of bacteria isolated from the air:water interface in the open environment have hydrophobic properties. A. Fattum and M. Shilo of the Hebrew University (personal communication) have found an interesting ecological correlation between cell-surface hydrophobicity and the partitioning of cells within bodies of water: benthic microorganisms (associated with bottom rocks or sediment) were found to adhere avidly to hydrocarbons, as studied here, whereas planktonic cells (unattached) were nonadherent. Similarly, in a recent study we have found that a high proportion (72%) of bacteria isolated from the surface of extracted teeth and dental matrices (incubated in the mouth of a healthy volunteer) adhered avidly to hexadecane (121).

These data, taken together with reports on the role of hydrophobic interactions in adherence and subsequent colonization of host cells (1,2,35a,59,54,78,80,86,95,103,104), in phagocytosis (23,113), in adherence to submerged surfaces (28,29,121), and in other phenomena of biological interest (13,16,18,20,25,26,32,44,48,57,61,66-69,84,85,89,105,109,120,121) suggest that bacterial hydrophobicity is a prime factor in bacterial adherence phenomena. This notion may have far-reaching consequences. For example, if hydrophobic interactions are responsible for adhesion of pathogens within the host, then mechanisms of preventing bacterial attachment to hydrocarbons may similarly remove cells bound to soft or hard tissues.

Adherence of bacteria to hydrocarbons can be dramatically influenced by growth conditions. These data suggest that affinity of the bacterial cell for hydrophobic surfaces may not always be to its advantage, and that the synthesis of the surface components mediating these adherence properties is carefully regulated. Moreover, following the dense colonization of a surface by adherent cells, a biological advantage may exist for the (temporarily) nonadherent cell which can return to the bulk aqueous phase in search of a more inviting habitat.

In the present work, we have demonstrated the importance of hydrophobic surface components in enabling the growth of RAG-1 on hexadecane. Adherence of RAG-1 to hydrocarbons may reflect a general microbiological phenomenon: A biological advantage is conferred upon microbial cells which can escape by means of hydrophobic surface components from the bulk aqueous phase to an interface which provides nutrition and enables growth.

SUMMARY

1. Two methods were developed during the course of this study: (i) bacterial adherence to liquid hydrocarbons, and (ii) replication of bacterial colonies onto polystyrene disks. The first method provided a quantitative general technique for measurement of cell-surface hydrophobicity on the basis of bacterial adherence to liquid hydrocarbons; in addition, the method was readily adapted for the isolation of a nonadherent mutant. The second technique enabled the simultaneous screening of large numbers of bacterial colonies for hydrophobic properties on the basis of adherence to the non-wettable plastic surface.

2. Adherence to hydrocarbon is not a specific property of hydrocarbon-degrading microorganisms, but is shared by other bacteria, such as strains of Serratia marcescens, Staphylococcus aureus, Streptococcus pyogenes, and various oral isolates.

3. Adherence to hydrocarbons is strain-specific: large differences were observed in the adherence of eight strains of Acinetobacter calcoaceticus to hexadecane.

4. Adherence to hydrocarbon is a function of growth medium and culture age. Exponential phase RAG-1 cells growing on complex medium were very poorly adherent, whereas cells harvested from the same medium in stationary phase adhered with high affinity to the test hydrocarbons.

5. An adaptation of the method employed for measuring bacterial adherence to hydrocarbon enabled the isolation of a nonadherent mutant, MR-481, which resembled RAG-1 in most properties, but failed to grow on hexadecane under specified conditions.

6. RAG-1 adherence was not specific for metabolizable substrate. RAG-1 adhered not only to hexadecane, which it can degrade, but also to hydrocarbons which it cannot metabolize, as well as to solid surfaces, such as epithelial cells and polystyrene. MR-481 did not adhere to these surfaces.

7. Adherence is a prerequisite for growth of RAG-1 on hexadecane under conditions of low initial cell density and limited emulsification of substrate. Addition of emulsifier (emulsan) enabled growth of nonadherent MR-481 cells on hexadecane. Thus, the mode of growth of microorganisms on liquid hydrocarbons (adherence or extracellular emulsification) depends largely on choice of microbial strains and growth conditions. The former mode of growth (i.e., via direct contact) is probably more closely related to growth on hydrocarbons in natural aqueous environments.

8. Prolonged incubation of MR-481 cells on hexadecane enriched for a population of partial revertant cells which regained partial adherence characteristics. Further growth of one such clone (RV-1) on hexadecane led to isolation of a second, more adherent clone, RV23-4. Both partial revertants were able to grow on hexadecane in liquid culture (as opposed to MR-481), but grew more poorly than wild type RAG-1 cells.

9. Thin fimbriae, approximately 3.5 nm in diameter, which were present on the outer surface of RAG-1, were absent from the surface of MR-481 cells, but reappeared in both partial revertant strains. Strain AB15, a non-agglutinating mutant which is also devoid of thin fimbriae, was nonadherent when grown on ethanol, but adherent when grown on complex medium. However, AB15 was unable to grow on hexadecane in liquid culture, even when the inoculum consisted of adherent AB15 cells.

10. Adherence of ethanol-grown RAG-1 cells to hexadecane was reduced by subjecting the cells to shearing. This treatment caused the release of a solitary antigenic component from the surfaces of wild type and partial revertant cells, but not from the surfaces of either thin fimbriae-deficient mutant, MR-481 or AB15.

11. A comparison of the properties of wild type cells with mutants MR-481 and AB15, and with partial adherence revertants RV-1 and RV23-4 led to the conclusion that thin fimbriae (i) are the main surface component responsible for the adherence characteristics of ethanol-grown RAG-1 cells, (ii) may be a crucial factor in enabling growth of RAG-1 on hexadecane, and (iii) constitute the major cell-surface agglutinogen of RAG-1. Adherence component(s) other than the thin fimbriae are present on cells grown in complex medium, or on hexadecane. Thus, while MR-481 lacks all the adherence components, mutant AB15 lacks only the thin fimbriae and exhibits partial adherence characteristics. Similarly, the partial adherence properties of strain RV-1 may be attributed to the reappearance of thin fimbriae.

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5. העשרה של חידקי RAG-1 אשר אינם נצמדים לפחמימן (ראה סעיף 1) הביאה לבידוד של המוטנט MR-481, אשר איבד את יכולתו להצמד לפחמימנים (97, 95). מוטנט זה דמה לזן הבר (RAG-1) ברוב תכונותיו, אך לא היה מסוגל לגדול על פחמימן (הקסאדקן) בהעדר תנאים אמולסיפיקטיביים. כאשר הוסף אמולסיפייר (אמולסן), המוטנט היה מסוגל לגדול על ההקסאדקן (97). ממצאים אלה מלמדים כי (א) הצמדות היא הכרחית לגידול על הקסאדקן בתנאים הדומים לאלה השוררים בטבע. ו- (ב) מערכות סגורות, אמולסיפיקציה של הפחמימן יכולה להביא לגידול של חידקים אשר אינם נצמדים היטב לפחמימן (97, 96).

6. הצמדות של RAG-1 אינה ספציפית מבחינת המשטח אליו הוא נצמד. חידקי RAG-1 נצמדים לא רק לפחמימנים אשר הם מסוגלים לפרקם (כגון הקסאדקן), אלא גם לפחמימנים אחרים, כגון אוקטן וקסילן (96, 94). כמו כן, RAG-1 נצמד גם לתאי אפיתל (95) ולפולי-סטירן (93). מוטנט MR-481 לא נצמד לאף אחד מהמשטחים האלה.

7. ציצונים (fimbriae) דקים אשר נמצאים על פני חידקי RAG-1, הינם חסרים במוטנט MR-481. הורדת הציצונים מעל פני תאי RAG-1 ע"י טיפול מכני מלווה בירידה בכושר הצמדותם להקסאדקן.
מוטנט אחר, AB15, אשר גם לו חסרים הציצונים האלה, לוקה בחלק מתכונות ההצמדות של הזן הבר. כמו כן, בודדו מתרבית של MR-481 שני רוורטנטים חלקיים, RV-1 ו- RV23-4, אשר קיבלו חזרה יכולת לגדול על הקסאדקן בתנאים ש- MR-481 אינו גדל. ציצונים דקים נמצאו על פני שני הרוורטנטים בכמות גדולה.
הממצאים האל מלמדים ש- (א) הציצונים הדקים על פני שטח RAG-1 גורמים לחלק מתכונות ההצמדות שלו, כפי שנחקרו כאן, ו- (ב) לציצונים האלה חשיבות ביכולתו של RAG-1 לגדול על הקסאדקן, בתנאים מסוימים.

8. האינטראקציה בין RAG-1 ובין הפחמימן עליו הוא גדל, משתקפת, בעקבות מחקר זה, כמקרה ספציפי של תופעה הרבה יותר כללית: דהיינו, ההידרופוביות של חידקים מסוגלת לסייע להם לברוח מהפאזה המימית אל משטחים עליהם יוכלו לגדול ולהתרבות.

ת ק צ י ר

תופעת ההצמדות של חידקים למשטחים שונים נחקרה רבות בעשור האחרון. במסגרת המחקר הנוכחי נעשה נסיון ללמוד אודות (1) הצמדות של חידקים למרכיבי-נפט (פחמימנים) נוזליים. (2) תפקיד אפשרי של הצמדות בגידול חידקים על פחמימנים, (3) הקשר בין הצמדות של חידקים לפחמימנים, ותופעות הצמדות אחרות. להלן המימצאים העיקריים של מחקר זה:

1. שתי שיטות פותחו במהלך המחקר: (א) שיטה למדידה כמותית של הצמדות חידקים לפחמימנים נוזליים (96, 94), ו- (ב) שיטה לבדיקה איכותית של הצמדות חידקים למשטח פלסטי הידרופובי (פוליסטירן) (93). הטכניקה הראשונה מהווה שיטה כללית וכמותית לבדיקת הידרופוביות של שטח פנים של מיקרואורגניזמים, המבוססת על יכולת של חידקים הידרופובים להצמד לטיפות הפחמימנים הנבדקים. פיתוח נוסף של השיטה הזאת אפשרה בידוד של מוטנט הלוקה ביכולתו להצמד לפחמימנים (97). השיטה השניה מאפשרת סקירה בו-זמנית של מספר גדול של מושבות חידקים על בסיס יכולתם להצמד לפוליסטירן.

2. הצמדות של חידקים לפחמימנים, כפי שהסתבר במהלך המחקר, אינה תכונה בלעדית לחידקים המפרקים פחמימנים, אלא משותפת למספר זנים שונים, אשר להם תכונות הידרופוביות (120, 96-94). יתר על כן, לא כל חידק המסוגל לפרק פחמימן הינו בעל יכולת להצמד אליו באפיניות גבוהה (96).

3. בבדיקה של שמונה זנים של Acinetobacter calcoaceticus נמצאו הבדלים גדולים ביכולתם להצמד לפחמימן (הקסאדקן), ממצא המראה שתכונת ההצמדות הנבדקת משתנה בין זנים שונים השייכים לאותו מין.

4. במחקר שבוצע על חידק מפרק נפט, A. calcoaceticus RAG-1, נמצא שתכונות הצמדותו לפחמימנים הינן פונקציה של (א) המצע עליו גדל החידק, ו- (ב) שלב גידולו (95). למשל, חידקים בשלב גידול אקספוננציאלי נצמדו פחות טוב מחידקים בשלבי גידול מאוחרים יותר. באופן כללי, חידקי RAG-1 אשר גודלו על מצע המכיל פחמימן כמקור פחמן ואנרגיה, או על מצע עשיר, נצמדו באפיניות יותר גבוהה מאשר חידקים שגודלו על מצע עני (כגון מצע מינימלי המכיל אתנול כמקור פחמן ואנרגיה).

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עבודה זו נעשתה בהדרכתם של פרופ'

יוג'ין רוזנברג ופרופ' דוד גוטניק

אוניברסיטת תל-אביב
המחלקה למיקרוביולוגיה

הצמדות של חידקים לפחמימנים ולמשטחים הידרופוביים אחרים

חבור לשם קבלת תואר "דוקטור לפילוסופיה"

מאת

מל רוזנברג

הוגש לסנאט של אוניברסיטת תל-אביב

אדר, התשמ"ב